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PHYSIOLOGICAL IMPLICATIONS OF HYDROGEN SULFIDE: A WHIFF EXPLORATION THAT BLOSSOMED

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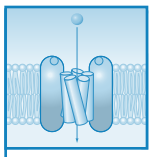


Wang R. Physiological Implications of Hydrogen Sulfide: A Whiff Exploration That Blossomed. *Physiol Rev* 92: 791–896, 2012; doi:10.1152/physrev.00017.2011.—The important life-supporting role of hydrogen sulfide (H_2S) has evolved from bacteria to plants, invertebrates, vertebrates, and finally to mammals. Over the centuries, however, H_2S had only been known for its toxicity and environmental hazard. Physiological importance of H_2S has been appreciated for about a decade. It started by the discovery of endogenous H_2S production in mammalian cells and gained momentum by typifying this gasotransmitter with a variety of physiological functions. The H_2S-catalyzing enzymes are differentially expressed in cardiovascular, neuronal, immune, renal, respiratory, gastrointestinal, reproductive, liver, and endocrine systems and affect the functions of these systems through the production of H_2S. The physiological functions of H_2S are mediated by different molecular targets, such as different ion channels and signaling proteins. Alternations of H_2S metabolism lead to an array of pathological disturbances in the form of hypertension, atherosclerosis, heart failure, diabetes, cirrhosis, inflammation, sepsis, neurodegenerative disease, erectile dysfunction, and asthma, to name a few. Many new technologies have been developed to detect endogenous H_2S production, and novel H_2S-delivery compounds have been invented to aid therapeutic intervention of diseases related to abnormal H_2S metabolism. While acknowledging the challenges ahead, research on H_2S physiology and medicine is entering an exponential exploration era.

I. INTRODUCTION

"Marge, what's wrong? Are you hungry? Sleepy? Gassy? Gassy? Is it **gas**? It's **gas**, isn't it?"

Dan Castellaneta (American Actor and Writer): *The Simpsons*

Victor Hugo's famous 1862 French novel *Les Miserables* set out an unforgettable scene when the hero Jean Valjean carries the unconscious Marius toward the smelly sewers of Paris where the unrelenting Inspector Javert lays in wait. As the fate of Jean Valjean has ever since turned into one of Broadway's showcases, the role of sewers and drains as the "intestine of the Leviathan" and the associated smelly gas, hydrogen sulfide (H_2S), attracted public attention at that time, as did a resonance of 1777 Paris accidents in which sewer gas emission led to several fatal cases. Hugo also described in his novel how he believed the people of Paris did not recognize that the noxious, odiferous waste that lay in the sewers of Paris was actually representative of their actions and lives and also that smelly waste could be reused and give value to people's daily lives as manure. Forward thinking for his time, he even described that what appeared to be horrid, smelly waste was actually the lifeblood of the people of his city and represents all aspects of humanity. In *Les Miserables* he wrote: "Those heaps of garbage at the corners of the the stone blocks, these tumbrils of mire jolting through the streets at night, these horrid scavengers' carts, these fetid streams of subterranean slime which the pavement hides from you, do you know what all this is? It is the flowering meadow . . . it is perfumed hay, it is golden corn, it is bread on your table, it is warm blood in your veins, it is health, it is joy, it is life" (254). Hugo was among the eminent novelists to give the sewer gas an actor role, but the description of H_2S as part of hepaticus (hepatic air) or one of the gases of putregaction by Johann Baptista van Helmont (1579–1644) (348) can be traced back in the 15th century, several hundreds of years before Hugo's time. The Italian physician Bernardino Ramazzini probably is the first one who described the toxicological effect of H_2S in 1713.

His observation centered on eye inflammation in workers who cleaned "privies and cesspits." Rammazzini attributed the eye irritation to a gaseous acid released when the workers disturbed certain areas of excrement. The linkage of rotten egg smell to H_2S was made around 1750 when Carl Wilhelm Scheele treated ferrous sulfide with a mineral acid, and he was rightfully entitled the first one to synthesize H_2S gas. Following studies by Lehmann in 1892 (347), Sayers et al. in 1923 (539) and Barthelemy in 1939 (29) extended the human study to animals for the toxicology of H_2S.

Hundreds of years of human knowledge on H_2S are nothing compared with H_2S-caused life destructions and extinctions on the earth over millions of years. H_2S is believed to be the root cause for multiple mass extinctions on earth, with one of the most significant in the end of Permian period. This extinction took place when the emissions from massive volcanic eruptions in Siberia caused a chain reaction of environmental events that resulted in the oxygen levels in the world's oceans to fall to extremely low levels. Subsequently, the depletion of oxygen caused a significant number of the species that lived in the ocean to die (686). Not all species were harmed though, as some non-oxygen (anaerobic) breathing organisms called green sulfur bacteria *(Chlorobium)* continued to grow and reproduce at high rates. These green sulfur bacteria used sulfate dissolved in water for respiration instead of oxygen, and subsequently produced H_2S. While the earth's life cycle continued, these organisms produced large amounts of H_2S and, from a theoretical perspective, created so much lethal gas in the ocean that it then diffused into the air and land destroyed the plant and animal life as well. By the end of the Permian period, 95% of marine species and 70% of terrestrial ones had vanished (686). Rock samples drilled in central China from the late Permian and early Triassic periods, which date to 252.2 million years ago, show several chemical signs of this catastrophe. In fact, some of the compounds extend back millions of years before the main extinction event. Known as biomarkers, these chemicals are evidence that green sulfur bacteria, *Chlorobium,* were living in the oceans (389).

Time is different now, but the H_2S-producing machineries or H_2S-utilization capacity remain within a great diversity of microorganisms (2, 353, 674). In this regard, bacteria are not alone. Certain plants process sulfate ion from the soil and convert it into plant protein. Decomposition of these plant proteins leads to the release of H_2S. This process also involves a wide variety of actinomycetes, fungi, and the bacteria heterotrophy *Proteus vulgaris*. Interestingly, plants are the primary producers of organic sulfur compounds and are able to couple photosynthesis to the reduction of sulfate, assimilation into cysteine, and further metabolism into methionine, glutathione, and many other compounds (352). The activity of the sulfur assimilation pathway responds animatedly to changes in sulfur supply and to environmental conditions that alter the need for reduced sulfur. Plants are able to incorporate inorganic sulfate, which is reduced to sulfide and is then incorporated into cysteine. This is in contrast to animals that have a dietary requirement for sulfur amino acids. Cysteine is the central intermediate from which most sulfur compounds are synthesized. The level of free cysteine in plants is low though the flux is considerably high, due to its rapid utilization for methionine, protein, and glutathione synthesis. Importantly, as anionic sulfate is the primary sulfur source for plants, it is relatively abundant in the environment. After being transported into roots, sulfate may stay there or be distributed to other parts. The transportation into cells is mediated by plasma membrane-localized hydrogen/sulfate cotransporters, driven by the electrochemical gradient established by the plasma membrane proton ATPase. Individual transporters show differing affinities for sulfate and are expressed in specific tissues. Some are strongly regulated at the mRNA level by changes in the sulfur nutritional status of the plant, although the exact function of each type of sulfate transporter is not yet fully understood. Among those life forms that share the H_2S-producting ability with bacteria and plants are invertebrates. Tissue homogenates of Manila clam *Tapes philippinarum* and the lugworm *Arenicola marina* produced significant quantities of H_2S gas (287). The worm *Urechis caupo Fisher also* produces H_2S (286).

H_2S had earned its fame mostly due to its unpleasant smell and fierce toxicity. From rotten eggs to sewage backup, H_2S reminds us of its existence. Our memory may still be fresh about the law suits and claims against the "Chinese dry wall" in the United States. It was claimed that once installed, these drywalls released H_2S at a rate above the minimum reported threshold odor level (0.5 ppb). H_2S may be released from inorganic sulfur compound or has its bacterial sources (244). Unpleasant to our noses, we know. H_2S-induced corrosion of appliances or copper plumbing or electrical wires, maybe. The long-term health abnormalities with this drywall-released rotten-egg gas for the residents, that remains to be determined. Accumulation of H_2S gas in confined or closed spaces, including septic tanks and cesspools, animal processing plants, pump mills, and sludge plants has been the cause of numerous cases of human deaths. Sudden release of H_2S gas in a huge amount from oil wells or refineries leads to infamous "knock-down" phenomenon of oilers and other petrochemical workers, an instant loss of consciousness often associated with respiratory failure. H_2S pollution or intoxication is a significant public concern, a major occupational hazard, and a toxicology focus (68, 578, 756). Medline search would have revealed ~19,500 publications related to "Hydrogen sulfide" up to the end of the last century, but few examined the physiological importance or a beneficial role of H_2S to our body.

If H_2S is so toxic or lethal to our body, why is it still produced in our body? The answer may reside in the stories of

another two gas molecules, nitric oxide (NO) and carbon monoxide (CO). NO was identified as an endogenous signaling molecule in the cardiovascular system initially, fulfilling a role of endothelium-derived relaxing factor (EDRF), a discovery rewarded with the 1998 Nobel Prize in Physiology or Medicine. Evidence has also been piled up for a signaling role of CO, produced in our body and involved in the regulation of physiological functions of multisystems (716). In this logic light, H_2S may also be important for the homeostatic control of our body, just like NO and CO did.

Production of H_2S in mammalian tissues has been known for a long time, but it was largely ignored as a metabolic waste. Significant H_2S levels were detected in mammalian tissues from human, rat, and cow in the range of 50–160 μM (213, 691). The enzymatic machineries for endogenous production of H_2S in mammals were also known, mostly composed of cystathionine β-synthase (CBS) (63, 491, 595), cystathionine γ-lyase (CSE) (7, 503, 742), and 3-mercaptopyruvate sulfurtransferase (MST) (175, 556, 654, 699). More in depth studies were called to demonstrate the physiological importance of H_2S beyond its mere presence in mammalian tissues.

Thanks to the occupational and toxicological studies on H_2S, we had been aware of many lethal and sublethal effects of exogenous H_2S on humans and animals. Kruszyna et al. (325) in 1985 reported that sodium sulfide, a donor of H_2S, augmented sodium nitroprusside-induced relaxation of guinea pig ileum. It also reversed the spasmolytic effects of azide and hydroxylamine on rabbit aortic strips. Acute and chronic NaHS intoxication of rats or mice had been reported to alter the content of certain amino acids and neurotransmitters in selective brain regions (317, 691, 692). "These effects of NaHS may indicate chronic low level H_2S neurotoxicity" (692) as originally thought. In vitro studies revealed that NaHS at toxicologically relevant concentrations had complex effects on electrophysiology properties of neuronal membrane and an array of K^+ conductance (316). In these occupational health or toxicology studies, cellular machinery that produces H_2S in vivo was not included in the research scope. How the endogenous H_2S production is regulated and what is the physiological effect of the endogenously generated H_2S were not studied. Whether the toxicological effects of NaHS bear physiological meaning or linked to endogenous H_2S level was not addressed.

A breakthrough in the effort in linking the endogenous H_2S level and functional changes came when Abe and Kimura (1) reported that H_2S donor, NaHS, facilitated the induction of hippocampal long-term potentiation (LTP) at micromolar concentrations. This effect is beneficial, away from the conventionally assumed toxic image of H_2S. They further confirmed the expression of CBS mRNA in the hippocampus using Northern blot. Pharmacological manipulation of CBS activity altered H_2S production correspondingly. Although it was not known by then whether altering endogenous H_2S level affects the LTP process, a "neuromodulator" role of H_2S in the brain was nevertheless suggested. The similar approach was employed to show H_2S was produced in vascular tissues and induced vasorelaxation (245).

Another breakthrough came in 2001 when the physiological role of H_2S was examined with a focus on CSE. Although CSE gene had been cloned from rat liver (165) and human liver (380), it was not clear whether the same CSE gene as that in liver existed in vascular tissues. Zhao et al. (773) for the first time cloned CSE gene from rat vascular tissues and demonstrated that both the expression and activity of CSE can be upregulated by NO, leading to increased production of H_2S from vascular smooth muscle cells (SMCs). The team also showed that, unlike NO that relaxes blood vessel walls by activating guanylyl cyclase to release cGMP, the specific molecular targets of H_2S in vascular SMCs are K_{ATP} channels. This is the first molecule target of H_2S identified in the cardiovascular system. By stimulating K_{ATP} channels, H_2S causes vasorelaxation within a physiologically relevant concentration range (773).

One way to obtain definite evidence for the physiological importance of endogenous H_2S is to eliminate the endogenous production of H_2S in a given organ or whole body and then monitor the phenotype change. Considering the dominant expression of CBS in the nerve system, CBS knockout mice would be of the choice to determine the impact of H_2S on neuronal functions or behavior. Homozygous knockout (KO) of CBS proves to be fatal to the mouse. Their life span would only be ~4 wk (694). Heterozygous knockout (HT) mice have a hyperhomocysteinemia phenotype. These mice have about twice the normal homocysteine level (694). Two major challenges for using CBS-HT mice in these studies are that endogenous H_2S production is still significant, and there is no solid evidence that the phenotype of mice shown is due to the lack of H_2S, rather than other metabolites affected.

The situation is different for CSE gene knockout. The research teams from Canada and the United States spent 5 years to develop CSE-KO mice and test their phenotype (738). This seminal work concluded in 2008 presents several major advancements in H_2S study. By eliminating most, if not all, H_2S production in cardiovascular system, CSE-KO mice develop hypertension with an onset at the age of 8 wk. This hypertensive phenotype is due to the lack of endogenous H_2S, since injection of exogenous H_2S into these mice rescues them from hypertension. Another important discovery out of this study is that hypertension development in CSE-KO mice is due to severe damaged endothelium-dependent relaxation of small resistance arteries.

Yang et al. (738) further demonstrated that vascular endothelial generates H_2S via CSE action. The stimulation of muscarinic cholinergic receptors on endothelium increases intracellular calcium and activated calcium-dependent calmodulin. The latter activates CSE and generates H_2S. H_2S acts on both endothelial cells and vascular SMCs to induce vasorelaxation. This whole chain process is disrupted in CSE-KO mice due to the lack of CSE. Thus H_2S can be characterized as another EDRF, joining the same campus with NO.

More evidence was obtained for the physiological role of H_2S ever since. Elrod et al. (160) demonstrated that cardiac-specific overexpression of CSE significantly limits mouse cardiac ischemia-reperfusion damage due to increased endogenous level. Altered expression of CBS and endogenous H_2S in vivo leads to many neurodegenerative diseases, and replenishment with exogenous H_2S reversed the pathology. Similarly, altered expression of CSE and endogenous H_2S levels were shown to be responsible for inflammation (755), atheroscleorosis (685, 690), diabetes (717), asthma (718), etc. By no coincidence, a role of endogenous H_2S in regulating human erectile dysfunction was recently demonstrated so that NO would not take the whole credit in relaxing human corpus cavernosum (136).

How does H_2S as a simple gas molecule interact with signaling proteins in our body? Choosing K_{ATP} channel complex as the target for study, Jiang et al. (279) showed that H_2S interacted with specific cysteine residues (Cys6 and Cys26) located on extracellular loop of SUR subunit of K_{ATP} channel complex. This interaction can be explained by a novel posttranslational modification mechanism, i.e., protein *S*-sulfhydration (420). H_2S induces covalent modification of cysteine residue by transferring its sulfhydryl group to cysteine residue of the concerned protein. *S*-sulfhydration usually enhances the activity of the modified proteins.

The identification of physiological importance of endogenous H_2S is a classical trudge of discovery, started from speculation, carried through with exploration, and realized with substantiation. Over the past decade or so, H_2S has undergone an image transformation from a feared and disliked smelly gas to an important endogenous signaling molecule that is now regarded as a key gas found in the body which most likely possesses powerful therapeutic use for many diseases.

Like NO and CO, H_2S fulfilled all of the criteria to define a gasotransmitter (681, 682). H_2S is a small gaseous molecule that is freely permeable to a membrane. H_2S is enzymatically generated in our body, and its endogenous metabolism is regulated. H_2S has specific physiological functions in different systems. The physiological effects of H_2S can be mimicked by exogenous H_2S donor at physiologically relevant concentrations. The cellular effects of H_2S do not need second messenger or specific cognate membrane receptors, but it triggers defined signaling cascade(s).

A third gasotransmitter in H_2S, after NO and CO, was born (681).

II. CHARACTERISTICS OF H_2S MOLECULE

A. Physical and Chemical Properties of H_2S

H_2S is a colorless and flammable gas. It has a molecular weight of 34.08 and a vapor density (d) of 1.19, heavier than air (d = 1.0). Its smell is characteristic of rotten eggs or the obnoxious odor of a blocked sewer. Its boiling point is -60.3°C, melting point is -82.3°C, and freezing point is -86°C. H_2S is the sulfur analog of water molecule and can be oxidized in a series of reactions to form sulfur dioxide (SO_2), sulfates such as sulfuric acid, and elemental sulfur.

Ambient air H_2S comes from two different sources. Organic sources include bacteria and decomposition of organic matters such as released from septic tanks, sewers, or water treatment plants. Inorganic sources are natural gas, petroleum refinery, rayon manufacturing, paper and pulp mill industry (225), sulfur deposits, volcanic gases, and sulfur springs. The air content of H_2S is conventionally expressed as ppm (parts per million) or ppb (parts per billion). One ppm of H_2S is equivalent to 1.4 mg H_2S/m^3. One mg of H_2S in 1 liter aqueous solution equals 717 ppm (standard temperature and pressure). H_2S can also be produced by reacting hydrogen gas with molten elemental sulfur at ~450°C. Hydrocarbons can replace hydrogen in this process.

The half-life of H_2S in air varies from 12 to 37 h. The presence of photoactive pollutants in ambient air and the variation of temperature would change the half-life of H_2S. In winter time, for example, the half-life of H_2S can be prolonged over 37 h in very cold and dry air. Temperature also affects the solubility of H_2S. At room temperature (20°C), one gram of H_2S will dissolve in 242 ml H_2O. Elevation of temperature increases H_2S' solubility. Over time, H_2S solution would turn cloudy due to the precipitation of elemental sulfur. This reflects the oxidation of H_2S in the solution. H_2S is a highly lipophilic molecule. At room temperature, 1 g of H_2S will dissolve in 94.3 ml absolute ethanol, or 48.5 ml diethyl ether. As such, H_2S easily penetrates lipid bilayer of cell membranes. However, NO and CO are more membrane permeable than H_2S due to greater lipophilic property of these two gases. This membrane permeability difference among the three gasotransmitters is also reflected by their dipole moments, with that of H_2S being 0.97, NO 0.16, and CO 0.13. H_2S also evaporates relatively easy from aqueous solutions (vapor pressure = 18.75×10^5 Pa). H_2S is a weak acid in aqueous solution with an acid dissociation constant (pK_a) of 6.76 at 37°C. It can dissociate into H^+ and hydrosulfide anion (HS^-), which in turn may dis-

sociate to H^+ and sulfide anion (S^{2-}) in the following reaction: $H_2S \leftrightarrow H^+ + HS^- \leftrightarrow 2H + S^{2-}$.

The definition of what constitutes free sulfide, as opposed to bound sulfide, should also be noted. Free sulfide is dissolved H_2S gas, which is a weak acid and in solution exists in the equilibrium $H_2S \leftrightarrow HS^- \leftrightarrow S^{2-}$. With the pK_{a1} ~7.0 and the pK_{a2} >17, there is essentially no S^{2-} in biological tissues, nearly equal amounts of H_2S and HS^- within the cell, and approximately a 20% H_2S/80% HS^- ratio in extracellular fluid and plasma at 37°C and pH 7.4. Due to the temperature sensitivity of pK_{a1}, the H_2S/HS^- ratio remains nearly constant in blood and tissues of ectothermic vertebrates over a wide range of body temperatures. For example, in trout blood at 10°C, the ratio is 15% H_2S/85% HS^-.

B. Intracellular H_2S Pools

H_2S, once produced in mammalian cells, can directly exert its biological effects via interaction with different signaling molecules, as occurs with the production of NO from NO synthases or CO from heme oxygenases. H_2S can also have its sulfur stored first and released later in response to a physiological signal. Acid-labile sulfur and sulfane sulfur are the two main forms of sulfur stores in mammalian cells (448, 449, 645). Ishigami et al. (268) used silver particles to measure free H_2S in neurons and astrocytes. They found that free H_2S level was low in basal conditions, which indicates that free H_2S, once produced, may immediately interact with its targets or absorbed and stored (268). Olson et al. (454) proposed a model in which constitutively produced H_2S diffuses from cytoplasm to the mitochondria where it is rapidly oxidized. The amount of H_2S being oxidized is therefore proportional to the oxygen partial pressure (Po_2) and biologically available H_2S is the net of production minus oxidation (454).

1. Acid-Labile Sulfur

Acid-labile sulfur pool releases sulfur atoms under acidic conditions from the iron-sulfur complexes of mitochondrial enzymes. In central nervous system, acid-labile sulfur has been measured as brain sulfide. The optimal pH for sulfur release from this store is <5.4. As the mitochondrial pH is between 7 and 8 and usually does not become acidic, it may be difficult for sulfur to be released from acid-labile sulfur pool under physiological conditions in the mitochondria. The technical challenge also made the determination of the size of acid-labile sulfur poor difficult since iron-sulfur complexes are unstable especially during protein denature treatment.

2. Bound Sulfane Sulfur

A pool of bound sulfane sulfur has been identified. After H_2S was released from rat brain homogenates under acid conditions, the addition of dithiothreitol (DTT), creating a reducing condition, to the resultant supernatant further released H_2S in almost the same amount as HCl alone did. DTT treatment also released more H_2S from Na_2S preabsorbed brain homogenates than without preabsorption. In contrast, acids did not release H_2S from Na_2S preabsorbed homogenates. It is rationalized that, under this experimental condition or others, H_2S may be released from a pool distinct from the acid-labile sulfur pool, which is reactive to reducing conditions (268).

Sulfane sulfur carries no charge. It is always attached to its carrier proteins via covalent bond between the S^o atom with its six valence electrons and other sulfur atoms. The existing forms of sulfane sulfur include thiosulfate ($S_2O_3^{2-}$), persulfides (R-S-SH), thiosulfonate (R-S-SO_2-R), polysulfides (RSnR, R is alkyl or aryl), polythionates (SnO_6^{2-}), elemental sulfur (S_8), and disulfides which have an unsaturated carbon adjacent to the C-S bond. A common S^o-binding domain has been found in 47 human genes (62), including albumin, rhodanese, CSE, and MST (645).

Exogenously applied free H_2S can be stored in cells as bound sulfane sulfur (268), as endogenously generated H_2S does. After labeled cysteine is injected into an animal, labeled bound sulfane sulfur is rapidly detected (138). This suggests that sulfur generated from cysteine metabolism is incorporated into different proteins as bound sulfane sulfur. This pool of sulfur releases H_2S under reducing conditions (448, 449). By reversibly binding sulfur to other molecules and releasing it later, sulfide can be stored or transported in a less labile, nongaseous state.

Bound sulfane sulfur pool is localized to the cytoplasm and releases H_2S under reducing conditions. Glutathione and cysteine are the major cellular reducing compounds. Their reducing capability is greater in alkaline conditions, which may explain the H_2S releasing from lysates of cultured neurons and astrocytes at a pH higher than 8.4 (268). Is this a redox-state related or simply reflects an alkaline-labile sulfur store? This question may be easily answered by conducting the same experiment but changing the reaction milieu to pH 8.4 while balancing the redox status. Moreover, the physiological implication of releasing H_2S from this store is questioned as it requires an alkaline microenvironment. To this point, one case may be made in mitochondria. From time to time, mitochondria may become alkali as the hydrogen pump works to different extents to move hydrogen across inner mitochondrial membrane (268). However, this scenario would not work unless the bound sulfane sulfur store is within mitochondria (268). Can this happen in cytosol? Alkalization of the cytoplasm may be possible in astrocytes by the high concentrations of extracellular K^+ that are normally present when nearby neurons are excited (268). This hypothesis would need to be tested, and whether it is a universal mechanism remains to be seen.

The absorption of free H_2S into the bound sulfane sulfur pool and its release are tissue type specific. Both the absorption and release of free H_2S are faster in liver and heart than in brain homogenates (268). The size of bound sulfane sulfur pool also depends on the H_2S producing activity. More H_2S-generating enzymes express and function, and more bound sulfane sulfur is formed. For example, the level of bound sulfane sulfur in cells expressing MST and CAT is about twofold higher than in the cells which express a defective mutant of MST, of which an active center cysteine 247 is replaced to serine and does not produce H_2S (426, 558). One explanation for this phenomenon is related to the H_2S producing activity of MST and the believing that H_2S produced by MST is stored as bound sulfane sulfur (308). As the activities of CBS and CSE are also influenced by the redox environment (28, 598), H_2S production, storage, and release would be lined together by redox balance.

Sulfane sulfur is most often quantified by cold cyanolysis and colorimetric measurement of ferric thiocyanate. Direct measurement of H_2S level using either gas chromatography analysis of head-space gas or methylene blue spectrometry method would not differentiate acid-labile sulfur from bound sulfane sulfur. Iciek and Wlodek (262) have shown that different methods used to release sulfur from bound sulfane sulfur pool may release sulfur from other sulfur pools.

C. Toxicology Profile of H_2S

The first defense line of human body to ambient H_2S intoxication is our olfactory response. Human sensing threshold for ambient H_2S odor is as low as 0.1–1 ppm. This value differs from one individual to another. Strong H_2S odor can be tolerated at 27 ppm, but eye irritation would already occur with 10 ppm H_2S. Acute exposure to 50–100 ppm H_2S leads to eye irritations (conjunctivitis, lacrimation, photophobia), neurological disorders (dizziness, headaches, loss of balance, lack of concentration, recent and long-term memory loss, mood unstableness, irritability, exhilaration, sleep disturbances), skin symptoms (itching, dryness, and redness), behavior changes (anger, depression, tension, confusion, fatigue, and vigor), general deficits (nausea, libido decrease, gastrointestinal tract upsets, loss of appetite), cardiovascular abnormalities (irregular heart beat or hypotension), and respiratory symptoms (apnea, cough, noncardiogenic pulmonary edema, and cyanosis). When ambient H_2S concentration reaches 100–150 ppm, the olfactory nerve is paralyzed after a few inhalations, and a sense of danger disappears while the real danger arrives (756). In fact, 320–530 ppm of H_2S leads to pulmonary edema with the possibility of death, while 530–1,000 ppm causes strong stimulation of the central nervous system and rapid breathing and then results in a loss of breathing. H_2S at $>$500 ppm can cause rapid unconsciousness and respiratory arrest. In the United States, ~125,000 employees in 73 industries are potentially exposed to H_2S. A survey of 10 years of data (1983–1992) from the Poison Control Centers National Data Collection system revealed at least 29 deaths and 5,563 exposures attributed to H_2S in the United States (578). Another occupational health study focused on a phenomenon called "knock-down" for petrochemical workers who were suddenly exposed to high concentration of H_2S in the working places. Among 221 cases reported, 14 ended with death during a 5-year period (1969–73), and a follow-up study of 250 workers' who claimed for H_2S exposure from 1979 to 1983 noted 7 deaths in Alberta, Canada. These deaths mostly related to the central nervous and respiratory systems while hepatic congestion and cardiac petechiae were also found (12). The drop in fatality rate (6% down to 2.8%) was attributed to improved first aid training and an increased awareness and appreciation of the dangers of H_2S.

Studies utilizing laboratory animals exposed to high concentrations of H_2S gas have yielded results similar to those observed in humans exposed at high level. For example, in one study, exposure to 1,655 ppm H_2S killed all five Sprague-Dawley rats within 3 min (377). While all male F-344 rats exposed to 500–700 ppm H_2S gas for 4 h died in another study, no rats died when exposed to concentrations up to 400 ppm under the same conditions (378). In the study by Beck et al. (40), all 10 male Wistar rats died after a 12-min exposure (mean) to 800 ppm H_2S. At concentrations of 335–587 ppm, H_2S exposure for 2–6 h caused death in 50% of animals tested (LC_{50}), including Sprague-Dawley, F-344, and Long Evans rats (497). Fewer deaths, however, were noted in approximately the same dose range in another study using F-344 rats (496).

Harmful cardiovascular effects have been noted after acute exposures to high concentrations of H_2S via exogenous inhalation (12). Reiffenstein et al. (509) exposed rabbits and guinea pigs to 72 ppm H_2S or intravenously injected NaHS. The animals developed ventricular extrasystoles, arrhythmias, altered cardiac contractility, and lower blood pressure. These changes could reflect a direct effect of H_2S on cardiovascular tissues or be consequent to neuronal and respiratory damage, which reduce oxygen supply to the cardiovascular system. Another animal study with NaHS intravenous injection led Baldelli et al. (25) in 1993 to believe that cardiovascular failure in the form of a profound hypotention was the cause of animal death, rather than central nervous system shutdown. These studies emphasized the importance of immediate cardiopulmonary resuscitation in treating H_2S "knockdown" exposures.

In a retrospective epidemiologic study using hospital discharge data from 1981 to 1990, Bates et al. (33) evaluated the H_2S toxicity to known target organ systems of residents of Rotorua, a New Zealand city that uses geothermal energy for industrial and domestic heating purposes. A signif-

icant increase in cardiovascular disorders was found among Rotorua residents, who were exposed to a range of H_2S concentrations from 20 to >400 $\mu g/m^3$ (33), compared with all other New Zealand residents. The interpretation of these data is limited, however, due to the lack of accurate data on H_2S exposure levels and the lack of control for potential confounding factors such as smoking and socioeconomic status.

The tolerance level of our body to H_2S varies depending on the H_2S exposure time. The lethal concentration for 50% of humans for 5 min exposure (LC50) is 800 ppm, and concentrations over 1,000 ppm cause immediate collapse with loss of breathing, with only a single inhalation needed. According to the Occupational Safety and Health Administration guideline for General Industry, the permissible exposure level to H_2S is "20 ppm ceiling for 10 min once, only if no other measurable exposure occurs." Recovery from acute intoxication is usually rapid and complete, though some symptoms may persist and some after-effects may not be able to be reversed. Multiple exposures and longer exposure time and the presence of other organo sulfur compounds as well will significantly increase the toxicity of H_2S. The United States Environmental Protection Agency has recommended a Chronic Reference Dose (RfD) of 0.8 micrograms per cubic meter of air, ~1 ppm, for both subchronic and chronic human inhalation exposure. At this concentration, no adverse health effects of H_2S should occur. Severe intoxication and even death have been reported in cases where there was a prolonged exposure over hours to 100 ppm H_2S and above.

The molecular mechanisms underlying the toxicological effects of H_2S are mostly attributed to mitochondrial poisoning (39, 124). Cytochrome *c* oxidase is the terminal enzyme in the electron transport chain in mitochondria that catalyzes the oxidation of ferrocytochrome C by molecular oxygen. By inhibiting cytochrome *c* oxidase, H_2S uncouples oxidative phosphorylation, and the production of ATP is subsequently decreased. This toxicological effect of H_2S on mitochondrial respiration is manifested at higher concentrations. It has been speculated that, on the other hand, H_2S at physiologically relevant level may stimulate mitochondrial consumption and ATP production.

Efforts have been made to identify suitable biomarkers for H_2S intoxication. H_2S in vivo can be oxidized to sulfate and thiosulfate and excreted in the urine. Very often urinary thiosulfate level has been used as a biomarker of H_2S exposure. Unfortunately, thiosulfate is not a specific indicator of H_2S intoxication. It will not differentiate ambient H_2S from normal dietary intake with high sulfur content, neither from endogenously generated H_2S. An increase in urinary thiosulfate level was observed in individuals exposed to 8, 18, or 30 ppm H_2S for 30–45 min (298). The urinary thiosulfate level peaked ~15 h after exposure. In a subject exposed to 18 ppm for 30 min, the peak urinary thiosulfate concentration at 15 h was 30 μmol/mmol creatinine. It returned to the same level as for nonexposed individuals 17 h later (mean concentration of 2.9 μmol/mmol creatinine). Measurement of blood sulfide level has also been proposed as a biomarker of exposure (272). However, this has limited clinical value because the blood samples must be collected within 2 h of exposure (272). As with the urinary thiosulfate level, a relationship between airborne H_2S level and blood sulfide level has not been established. Jappinen and Tenhunen also investigated the use of alterations in blood heme metabolism as a possible biomarker of H_2S exposure (272). The activities of the enzymes for heme synthesis, i.e., δ-aminolaevulinic acid synthase (ALA-S) and heme synthase, were examined in 21 cases of acute H_2S toxicity in Finnish pulp mill and oil refinery workers. These people were exposed to 20–200 ppm H_2S for periods ranging from ~1 min up to 3.5 h. Several subjects lost consciousness for up to 3 min. Activities of ALA-S and heme synthase were decreased after exposure to H_2S, though changes in heme metabolism were not.

III. THE SMELL OF LIFE: H_2S AND EVOLUTIONARY BIOLOGY

A. Life Extinction and Life Survival

Historically and in the present day, evolution biologists and toxicologists know plenty about the downside of H_2S. During the Permian period millions of years ago, the Earth experienced truly devastating consequences to the depletion of oxygen and massive accumulation of H_2S in the oceans and atmosphere to such large quantities that the gas ended up turning the sky green and choked off oxygen for plants, animals, and marine life (686). This excess H_2S condition killed more than 90 percent of life species. Back to World War One, H_2S was used by the British as a chemical agent. It was not considered to be an ideal war gas, but while other gases were in short supply, it was used at two separate wartime events in 1916 (188). Even now, H_2S is a force to be reckoned with. This gas is considered a broad-spectrum poison to humans in the nervous system, respiratory system, and cardiovascular system. The toxicity of H_2S is comparable to that of hydrogen cyanide due to the fact that it creates a complex bond with iron in the mitochondrial cytochrome enzymes, and subsequently prevents cellular respiration.

In sharp contrast to its toxic image, the potentially lethal molecule H_2S is also made in human body naturally, although at much lower concentrations. H_2S appeared in mammalian blood from nanomolar to micromolar concentration range. This appearance of H_2S in mammalian body may remind us of some of the earliest microbes and even a few living today who can solely rely on sulfur, and not oxygen, to obtain energy from metabolism (686). H_2S is of

physiological importance as it actually relaxes blood vessels (773). While NO does most of the vessel-relaxing work in large vessels, H_2S may be responsible for similar action in smaller blood vessels (684). In Alzheimer's disease, the brain's H_2S concentration was found to be severely decreased, which led to a theory that H_2S may be be the limiting factor for the development of this disease (167). H_2S was also discovered to exhibit protective effects against cardiac ischemia. It was also found that H_2S, like NO, is involved in the relaxation of smooth muscle that causes erection of the penis, presenting possible new therapy opportunities for erectile dysfunction (136). Another potential medical use for the deadly gas is to create a suspended animation, which may save millions of lives someday in the future. Administration of H_2S to mice has been shown to slow the creature's metabolism and induce a suspended state of animation (56). For details of the life-saving roles of H_2S, readers are referred to sections below. It suffices to say that too much H_2S would cause life extinction but without the gas life would not survive either. "We are what we smell" (683).

B. H_2S Metabolism in Bacteria

H_2S was one of the earliest products of bacterial decomposition to be recognized (118). Bacterial production of H_2S was first described in 1895 by Orlowski (460) and later became an archetype to distinguish paratyphoid from enteritidis groups in 1915 by Burnet and Weissenbach (81). From earlier on in 1912–1923, it was realized that not only cystine (79) but also thiosulfate ($S_2O_3^{2-}$) (638, 639) or sulfite (712) could provide fuel for H_2S production in bacteria. The enzymatic process for H_2S production from cystine and cysteine was unearthed by Tarr in 1933 (627, 628) in *Proteus vulgaris* and *Chromobacterium prodigiosum*. He later showed that the production of H_2S by *Proteus* from cysteine and thiosulfate was additive, independent, and caused by two distinct enzyme systems (629). Desnuelle and Fromageot in 1939 isolated cysteinase from *Bacterium coli* (142). Fromageot concluded in 1951 that cysteinase occurs in all bacteria that produce H_2S from organic media, and this process requires the reduction of cystine to cysteine (192).

1. Sulfate- and Sulfur-Reducing Bacteria: Where H_2S is Produced

A) SULFATE-REDUCING BACTERIA. Sulfate-reducing bacteria (SRB) propulgate in a pH range of 5.5 to 8.5 and dwell in temperatures ranging from 0 to 100°C, with their optimum range between 24 and 42°C. These anaerobes are considered among the most ancient forms of bacteria that use sulfate as the terminal electron acceptor of their electron transport chain to produce energy. This process is named dissimilatory sulfate reduction (634). The whole reduction process is composed of eight steps of electron transport. Although the intermediate products are not easily detected, hydrogen or organic molecules are oxidized in the absence of oxygen, and H_2S is created as the final product. As such, the sulfurous odor is often a signpost for the presence of these bacteria in nature, and in organic-rich marine sediments, the production of H_2S by bacterial reduction of dissolved sulfate is a predominate progression of anaerobic respiration (72).

Sulfate entry into the cytoplasma of SRB is the first step of the reduction process as all enzymatic steps leading from sulfate to sulfide occur in the cytoplasm or in association with the inner side of the cytoplasmic membrane (446). Hence, sulfate has to be carried into the cell. Sulfate is concurrently transported with three protons in the freshwater species of *Desulfovibrio desulfuricans* and *Desulfobulbus propionicus* (132, 133), and is driven by a proton gradient. Sulfate uptake in moderately salt-dependent species (*Desulfovibrio salexigens* and *Desulfococcus multivorans*) is cotransported with 3 Na^+, driven by a sodium ion gradient (322). Taking account of the efflux of H_2S as a neutral product, sulfate transport is electrogenic under these conditions. However, an electroneutral process has also been proposed, involving an H^+/Na^+ antiporter that moves one sulfate with two H^+ or Na^+ across the cytoplasmic membrane of SRB (322, 656).

Most of the SRB described to date belong to one of the five phylogenetic lineages. The first encompasses the mesophilic proteobacteria with the genera *Desulfovibrio, Desulfobacterium, Desulfobacter,* and *Desulfobulbus*, all in the delta subgroup (299). The second involves the thermophilic Gram-negative bacteria with the genus *Thermodesulfovibrio*, while the third is the Gram-positive *Peptococcaceae* with the genus *Desulfotomaculum*. A genus of *Archaea* named *Archaeoglobus* is the fourth lineage (97). Finally, the fifth lineage, the *Thermodesulfobiaceae*, has been recently reported (410). Due to the fact that dissimilatory sulfate reduction is inhibited under oxic conditions, SRB can grow at the expense of sulfate reduction only when there is absolutely no molecular oxygen present. Therefore, SRB are considered to be strictly anaerobic microorganisms and are, for the most part, found mainly in sulfate-rich, anoxic habitats (131, 171). These conditions apply in marine sediments due to the fact that ocean water is rich in sulfate, with its concentration recorded as high as 30 mM. SRB also exist in freshwater sediments, where the sulfate concentration is often far below 1 mM but is stable at this level due to the reoxidation of H_2S to sulfate at the oxic/anoxic interface. This is caused by the active nature of chemolithotrophic and photolithotrophic bacteria (242). As many SRB have been seen to utilize electron acceptors other than sulfate, they can also be located in anoxic habitats depleted of sulfate, such as in the human intestinal tract (634).

B) SULFUR-REDUCING BACTERIA. Sulfate (SO_4^{2-}) is the highest oxidation state of sulfur, and elemental sulfur is the lowest oxidative state. The elemental sulfur is thought to be the most prevalent sulfur species in sediments and geological deposits. A variety of prokaryotes have been recorded as being able to reduce elemental sulfur (59, 484, 596), manganese (IV) (423), iron (III) (86), or other lower oxidation states of sulfur as electron acceptors, but not sulfate. *Desulfurmonas* acetoxidans represent the true dissimulatory sulfur-reducing bacteria. These bacteria have a nearly obligate and specific requirement for elemental sulfur or disulfides such as cystine or reduced glutathione as the terminal electron acceptor under anaerobic conditions. They are unable to utilize the electron acceptors reduced by the sulfate-reducing bacteria, such as sulfate (SO_4^{2-}), sulfite (SO_3^{2-}), thiosulfite ($S_2O_2^{2-}$), or other oxidized sulfur or nitrogen compounds. It is also important to note that acetate is a suitable carbon substrate for *Desulfurmonas*. In contrast to dissimilatory sulfate reduction for sulfate-reducing bacteria, some sulfur-reducing bacteria, such as facultative anaerobes, can grow with O_2 while others are strictly anaerobic. Among the sulfate-reducing bacteria, only a few species have been recorded as having the ability to grow and thrive with elemental sulfur (54). Other sulfate-reducing bacteria can distribute small amounts of H_2S in a by-reaction which does not support growth when transferred from sulfate-grown cultures to media with crystalline (rhombic) or colloidal sulfur. Sulfur can even limit germination of many species of sulfate reducers (23, 24, 80). This effect is most likely induced by the pro-oxidant nature of elemental sulfur which shifts the potential of redox couples in the medium and cells to halt bacterial proliferation. Some subtypes of sulfate-reducing bacteria can also use sulfur as an alternative electron acceptor in the absence of SO_4^{2-} or other appropriate electron acceptors.

Incomplete oxidation of organic substrates in sulfur-reducing bacteria, such as *Sulfos pirillum*, *Wolinella*, *Shewanella,* and *Pseudomonas mendocina* will produce acetate (614). On the other hand, the complete oxidation in bacteria like *Desulfofuromonas* or *Desulfurella* will have CO_2 as the final product. While bacterial sulfur reducers may be mesophilic or moderately thermophilic, archaeal sulfur reducers are mainly thermophilic (436). The most common habitats of the hyperthermophilic sulfur reducers tend to be solfataric fields, hot springs, and hydrothermal systems in the deep sea, whereas mesophilic bacterial sulfur reducers can be isolated from almost every freshwater or marine sediment. Different from sulfate reduction, the reduction of the lower oxidation states of sulfur may only serve as hydrogen sinks for a "facilitated fermentation." Overall, these processes vary and make up a scope ranging between true sulfur respiration and sulfur reduction as a simple by-reaction. For example, a freshwater *Beggiatoa* was found to diminish stored sulfur under anoxic conditions with added acetate (434).

2. *Sulfur-Oxidizing Bacteria: Where H_2S Is Consumed*

Biological oxidation of H_2S to sulfate is one of the major reactions of the nature sulfur cycle. Reduced inorganic sulfur compounds, referred to as sulfur, are exclusively oxidized by phylogenetically diversified prokaryotes, and sulfate is the major oxidation product. Lithoautotrophic bacterial endosymbionts carry out sulfur oxidation in members of the *Eukarya* (433). In the domain *Archaea*, aerobic sulfur oxidation is limited to members of the order *Sulfolobales* (385), whereas aerobic lithotrophs or phototrophs are in charge for sulfur oxidation in the domain *Bacteria*.

Aerobic sulfur-oxidizing prokaryotes belong to genera like *Acidianus*, *Acidithiobacillus*, *Aquaspirillum*, *Aquifex*, *Bacillus*, *Beggiatoa*, *Methylobacterium*, *Paracoccus, Pseudomonas*, *Starkeya*, *Sulfolobus*, *Thiothrix*, *Thioploca* (710), *Thermithiobacillus*, *Thiobacillus, Thiomicrospira* (328), and *Xanthobacter* and are mainly mesophilic (191). Some of these lithotrophic bacteria can utilize polythionates, but others cannot. Anaerobic phototrophic sulfur-oxidizing bacteria are, for the most part, neutrophilic and mesophilic and belong to genera like *Allochromatium*, which was previously classified under formerly *Chromatium*, *Chlorobium, Rhodobacter, Rhodopseudomonas, Rhodovulum*, and *Thiocapsa* (191).

The nonphototrophic obligate anaerobe *Wolinella succinogenes* oxidizes H_2S to polysulfide while undergoing fumarate respiration. Prokaryotes oxidize H_2S, sulfur, sulfite, thiosulfate ($S_2O_3^{2-}$), and various polythionates under alkaline, neutral, or acidic conditions. Lithoautotrophic growth in the dark has been detailed for *Thiocapsa roseopersicina, Allochromatium vinosum*, and other purple sulfur bacteria, as well as for purple non-sulfur bacteria like *Rhodovulum sulfidophilum*, formerly *Rhodobacter sulfidophilus*, *Rhodocyclus genatinosus*, and *Rhodopseudomonas acidophila* (318, 564). Sulfur oxidation by methylotrophic bacteria has also been seen from growth with methylated sulfur compounds, such as dimethyl sulfide (135, 609).

Sulfur reduction and sulfur oxidation are handled by two different groups of bacteria, but both contribute to a balanced H_2S level in a given environment **(FIGURE 1)**. Take the Chilean and Peruvian shelf or the Namibian shelf and slope as a study case where the highest rates of bacterial sulfate reduction appear to occur right below the sediment-water interface. However, H_2S is hard to detect there because most sulfide is reoxidized within the sediment and fluxes of H_2S within the water column are mostly insignificant (173, 174, 187, 332). Therefore, with the exception of some coastal embayments, fjords, marginal seas, and estuaries with limited water interchange and strong stratification, H_2S rarely accumulates in the overlying water column. In short, the retention of H_2S is decided by three factors: bacterial or chemical oxidation, precipitation as iron sulfides, and formation of diagenetic organic sulfides (71). Ferdel-

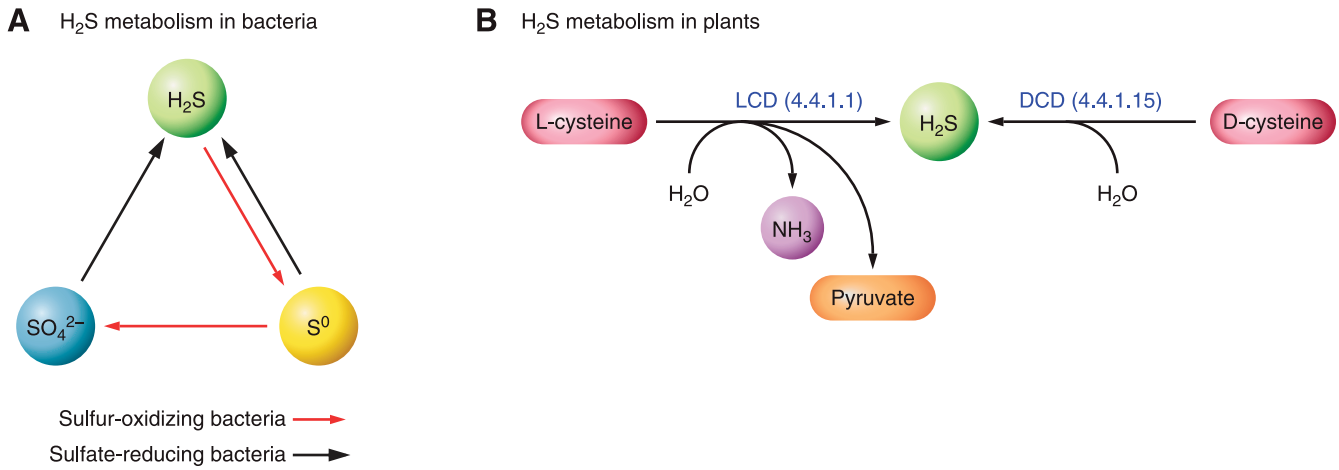


FIGURE 1. H_2S metabolism in bacteria and plants.

man et al. (174) showed that 90% of the total oxygen uptake in the organic-rich marine sediments from the continental slope of Namibia was due to the oxidation of H_2S. The study supports the finding that H_2S production is important not only for the benthic mineralization of organic matter but also as a sink for dissolved oxygen in the water column. Oxygen demand for the oxidation of dissolved sulfide represents the second most important sink after aerobic respiration. Accumulation of dissolved H_2S asks for more sulfide oxidation, which would lead to depletion of dissolved oxygen in the bottom water, negatively affecting fisheries and benthic marine life (143).

Purple sulfur bacteria, members of the families *Chlorobiaceae* and *Chromatiaceae*, are phototrophic obligate aerobes that are found in waters with high H_2S concentrations. Purple sulfur bacteria and green sulfur bacteria use H_2S as electron donors, oxidizing it to elemental sulfur or to sulfate by using dissolved oxygen, metal oxides (for example, Fe oxyhydroxides and Mn oxides), or nitrate as the oxidant (435).

Large sulfur-oxidizing bacteria often cover the seafloor in mats within organic-rich coastal areas, at hydrate ridge methane seeps, at hydrothermal vents, on whale falls, and in coastal upwelling regions. The presence and function of these large sulfur bacteria contribute to the overall sulfide oxidation in the environment. For example, ~55% of total sulfide oxidation in Namibia is undertaken by the large sulfur-oxidizing bacteria (174). The closely related genera *Beggiatoa*, *Thioploca*, and *Thiomargarita* are among the largest prokaryotes that have been recorded, and they often harbor a vacuole that makes up for 90% of the cell volume. These large sulfur-oxidizing bacteria, who dwell on the sea floor, perform a key ecological function by not allowing the release of toxic H_2S from the sediment into the water column. These bacteria often live in transition zones, between aerobic and anaerobic conditions, where both molecular oxygen and H_2S are found (415). The capacity of these environments to retain and oxidize sulfide also depends on the abundance and activity of the large sulfur bacteria. For example, *Thioploca* spp. is abundant on the Chilean and Peruvian shelf, but not on the Namibian shelf (253), while *Beggiatoa* spp. established significant populations only at stations on the outer portion of the Namibian shelf.

The presence of sulfide-oxidizing bacteria that couple the oxidation of sulfide to the reduction of nitrate to ammonium has provided another line of inquiry into the benthic sulfur cycle (463). Large sulfur-oxidizing bacteria can oxidize sulfide as long as they maintain an intracellular reservoir of dissolved nitrate. When experiencing anoxia, these large vacuolated *Beggiatoa*, *Thioploca*, and *Thiomargarita* respire nitrate, which can be concentrated up to 10,000-fold (500 mM) within the bacteria's intracellular vacuoles (545). As such, in the absence of external oxidants, these bacteria can use the intracellular storage of oxidizing power as a means of survival. A net H_2S flux across the sediment-water interface of the coastal upwelling system off Namibia has been recorded that is ascribed to high bacterial sulfate reduction rates near the sediment surface which cannot be compensated by the capacity of the sediment to oxidize and precipitate sulfide (72). The water column H_2S level would also be elevated by the methane eruptions or rising of methane bubbles, which promotes episodic advective transport of H_2S from the methanogenic zone (300).

C. H_2S Metabolism in Plants

H_2S concentrations in plants and the amount of H_2S released by plants have been reported. For example, H_2S concentrations in *Arabidopsis thaliana* leaf vary from 1 to 15 μM. Younger leaves and leaves from younger plants contain higher H_2S concentrations than older leaves and older plants (513).

The production of H_2S by plants, for the most part, stems from cysteine metabolism. Therefore, cysteine-synthesizing and degrading enzymes, such as *O*-acetyl-L-serine (thiol)

lyase (OAS-TL) (EC 4.2.99.8), L-cysteine desulfhydrase (EC 4.4.1.1), and 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) are closely related to H_2S production in plants (515). OAS-TL, also called cysteine synthase, catalyzes the formation of cysteine from *O*-acetyl-L-serine (OAS) and sulfide. During the process, H_2S can be released as a side reaction. The compartmentalization of cysteine as well as H_2S inside plant cells, the cytosol, plastids, and mitochondria, is closely linked to the corresponding intracellular localization of OAS-TL (381, 468). Nifs/NFS is also potentially involved in H_2S production in *Arabidopsis* for its L-cysteine desulfhydrase-like activity (349).

L-Cysteine desulfhydrase (L-CD) was first reported in 1980 (231). It specifically metabolizes L-cysteine to produce H_2S, pyruvate, and ammonium (9). L-CD expression and activity can be upregulated by pathogen attack. As such, this enzyme could be a key factor in releasing H_2S during a plant defense reaction (58, 508). Whether or not the quantity of H_2S released from the host is toxic to the pathogen is dependent on its accumulation at the site of pathogen attack and on the pathogen's capability to metabolize H_2S. Also producing H_2S, D-cysteine desulfhydrase (D-CD) only decomposes D-cysteine, not L-cysteine (430, 468) **(FIGURE 1B)**. Riemenschneider et al. (515) identified a gene encoding a putative D-CD protein in *Arabidopsis thaliana*, based on high homology to an *Escherichia coli* protein called YedO that has D-CD activity. The deduced 43.9-kDa *Arabidopsis* protein consists of 401 amino acids. Hydroxylamine and aminooxyacetic acid at low micromolar concentrations inhibit D-CD. While D-CD protein level remains constant in plants over different growth phases, its activity is low in the proceeding development of *Arabidopsis* but high in senescent plants. The dissociation of D-CD expression and D-CD activity is also shown by the regulation of D-CD expression without corresponding changes in D-CD activity when the plants are grown under low sulfate concentrations (515).

Sulfur is one of the critical macronutrients for the plant life cycle as it can affect crop yield, plant growth, and vigor. Sulfur fertilization has been known to facilitate recovery from or even increase resistance against, pathogens in plants, referred to as the so-called sulfur-induced resistance (SIR) (468). Increased atmospheric H_2S concentrations exert significant effect on thiol metabolism in plants and that H_2S exposure (0.25–0.75 ml/l) for a short time (3–48 h) increases the content of cysteine by 20-fold and glutathione by 4-fold in *Arabidopsis thaliana* (515). Comprehending the protective effects of SIR is useful for a full understanding of the role of endogenous sulfur-containing defense compounds (SDCs). SDCs embody glucosinolates, phytoalexins, elemental sulfur, glutathione, phytochelatins, various secondary metabolites, and sulfur-rich proteins. SDCs also have an important part to play in the viability of plants under biotic and abiotic stress. Their constitutive and/or stress-induced formation is dependent on demand-driven sulfate uptake and assimilation. H_2S may also belong to the group of SDCs, but this is being debated (508).

H_2S can also be formulated through the reduction of sulfate in plant. After transportation of sulfate into the plastid, sulfate can bind to ATP to form adenosine-5′-phosphosulfate (APS). Most of the APS is reduced to sulfide through the enzymes APS-reductase (APR) and sulfite reductase (241). Among the factors that increase the activity and steady-state mRNA level of APS reductase are sulfate starvation, oxidative stress, or heavy metal exposure (235). This upregulation serves as a sulfur homeostatic mechanism to maintain the redox balance. Increased oxidative stress would require increased levels of cysteine, glutathione, and phytochelatin for countering acts.

The role of H_2S metabolism in plants has attracted attention in recent years, but the progress in defining the metabolic pathways of H_2S in different developmental stages of plants and their alterations under various environmental perturbations has been limited. The improved root organogenesis and seed germination by exogenous H_2S treatment have been reported in several plants (95, 760). H_2S fumigation also improves the freezing tolerance of wheat shoots (602) and protects plants from toxication of copper and chromium as well as other osmotic stresses in different species (759–761). A recent study showed that the mRNA levels of L-CD and D-CD in *Arabidopsis* gradually elevated in a developmental stage-dependent manner. Furthermore, the transcriptional expression of of these two cysteine desulfhydrases was significantly higher in stems and cauline leaves than in roots, rosette leaves, and flowers (283). After withholding water supply to the plant for 14 days, the expression of L-CD and D-CD was significantly upregulated and the production rate of H_2S from these plants increased approximately six- to sevenfold. Rewatering the soil to completely wet for 1 day saw the transcriptional expression of L-CD and D-CD in the plant reversed, and endogenous H_2S production rate dropped, to the predrought level. The dehydration-induced changes in the expression of drought marker genes (*DREB2A*, *DREB2B*, *CBF4,* and *RD29A*) followed the same pattern as that of L-CD and D-CD genes. Finally, NaHS fumigation (80 μM, 6 h every other day) during the drought period stimulated further the expression of drought-associated genes. Without NaHS treatment, most control plants after 2 wk of drought died, but most NaHS-treated plants survived. After rewatering, seedlings of NaHS-treated plants had a higher survival rate of 80% than the 20% survival rate of nontreated plants. This study indicates that the variation of endogenous H_2S level may regulate the expression of drought-associated genes in the plant, and increasing H_2S fumigation may enhance drought resistance of *Arabidopsis* and potentially that of other plants (283). The effect of H_2S on stomatal closure has been a controversial topic. Jin et al. (283) showed that seedlings of NaHS-treated plants displayed a significant reduction in

the size of the stomatal aperture. This observation is consistent with a previous report that exogenous H_2S released by its donors induced stomatal closure in different plant species (95). In contrast, another study reported that exogenous H_2S donors (NaHS and GYY4137) caused stomatal opening in the light and prevented stomatal closure in the dark. These H_2S donors also reduced NO accumulation in guard cells of *Arabidopsis thaliana* (375). The reasons for these different observations are not clear.

D. H_2S Metabolism in Invertebrates

Similar to plants, invertebrates also have the ability to produce and process H_2S. Sedentary invertebrates are prolific in many sulfidic environments, and often make up the majority of the macrofauna (285). The behavior of such animals, which includes bivalves, tube worms, and some burrow-dwelling animals (176), is composed primarily of rhythmic muscular movements for feeding and gas exchange. To determine whether macrofaunal invertebrates have the capacity to produce H_2S, H_2S production in tissue homogenates of the Manila clam *Tapes philippinarum* and the lugworm *Arenicola marina* were measured (287). With the addition of 10 mM L-cysteine and 2 mM pyridoxal-5′-phosphate, significant quantities of H_2S were detected in tissue homogenates from both animals. Aminooxyacetic acid (AOA) abolished H_2S production in the Manila clam tissues, but only inhibited H_2S production by half in the body wall of the lugworm, indicating the critical role of CBS in H_2S production in the Manila clam but only being partially responsible in the lugworm. However, even without L-cysteine and pyridoxal-5′-phosphate, tissues from the Manila clam still produced measurable H_2S. Furthermore, the addition of the second thiol substrate (2.5 mM 2-mercaptoethanol) doubled the H_2S production in tissues of Manila clam, but not in the lugworm. These results suggest that CBS activity in Manila clam may not entirely rely on the cofactor pyridoxal-5′-phosphate or only use L-cysteine as the substrate. Alternatively, an "activated serine sulfhydrase pathway" may function in the clam as discovered previously in certain microfauna. *Urechis caupo* is a large (>60 g) worm that inhabits U-shaped burrows in mudflats along the California coast. The homogenate of *U. caupo* body wall tissue also produces H_2S upon addition of cysteine (286).

In contrast to extended works on H_2S metabolism in mammalian smooth and cardiac muscle, little information about potential signaling actions of H_2S in invertebrate muscle has been published. H_2S at concentrations above 5 mM inhibited body wall muscle contraction in *U. caupo* (286). While this concentration is clearly toxic, at lower concentration of 0.05–0.1 mM, H_2S impaired or prevented associative learning and long-term memory in the freshwater snail *Lymnaea*, but the effects of H_2S at this concentration range on muscle tone were not investigated (286). The 5-hydroxytryptamine-preconstricted branchial muscles of the clamp *M. mercenaria* was further constricted by NaHS at concentration as low as 10 nM. The same proconstrictive effect on the same tissues was also observed with NO (197). Differently, sodium nitroprusside (SNP), a NO donor, alone has no effect on the contractility of the body wall muscle of *U. caupo*, but NaHS alone contracted this muscle. SNP further potentialed NaHS-induced muscle contraction (286). It was speculated that the interaction of NO and H_2S may form *S*-nitrosothiol complexes so that the effect of H_2S is altered. Alternatively, NO might precondition the H_2S targets to render higher H_2S sensitivity (286).

E. Tracing Down H_2S Along the Universal Phylogenetic Tree

The "Universal Tree of Life," based on rRNA sequences, shows abundant thermophilic sulfur-respiring organisms near its root, particularly in the Archaea (178, 465). These archaebacteria are the most closely related of all prokaryotes to the eukaryotic nucleocytoplasm. They may even represent the ancestral eukaryotic phenotype. The challenges to this evolutionary continuity theory are the fact that modern eukaryotic cells are not thermophilic (70) and whether eukaryotic cells are still sulfur-respiring organisms. Especially intriguing is the observation that some bacteria are mesophilic or moderately thermophilic while they possess sulfur reducing power. Sulfur reduction is found among prokaryotes (492, 709), and certainly the conventional belief that eukaryotes do not have sulfur-reducing capacity would not hold true any longer.

Elemental sulfur reduction to H_2S by animal cell extracts was reported in as early as 18th century by de Rey-Pailhade (134). McCallan and Wilcoxon in 1931 (397) found that fungi or plant leaves also reduced elemental sulfur to H_2S when elemental sulfur was used as a fungicide, which was interpreted as a nonenzymic reaction between elemental sulfur and reduced glutathione (GSH) (531). Human is another species walking along the same trail. In his 1939 review, Comroe (123) noticed that patients with rheumatoid arthritis were deficient in sulfur and recommended supplements of elemental sulfur as a therapeutic avenue. On the other hand, Monaghan and Garai in 1924 (409) noticed the symptom of H_2S poisoning when the ointments containing elemental sulfur were applied to skin. In one study, a man was injected intravenously with colloidal sulfur and within seconds H_2S was detected in his breath (409). As the reaction happened too quickly to involve intestinal bacteria, the observation seemed to suggest that elemental sulfur reduction can occur in human tissues. Erythrocytes can reduce elemental sulfur to HS^- using reducing equivalents obtained from glucose oxidation (548). Not only glucose, other electron carriers such as NADH and NADPH may also help sulfur reduction. In fact, NADH, NADPH, and GSH all stimulate H_2S production in cellular lysates.

More evidence on a possible eukaryotic-Archaea relationship in terms of sulfur reduction has arrived. Eukaryotic genomes contain sequences of disparate evolutionary origins, suggesting origin from a fusion of archaebacterial and eubacterial cells (220, 390). For example, some metabolic enzymes in eukaryotes are the same as those in archaebacteria, such as the vacuolar H^+-translocating ATPase (211).

Sulfate reduction and sulfur reduction could differ greatly in this evolutionary context, as plants reduce and assimilate sulfate in the plastids (74), not in cytoplasm. In addition, sulfate reduction in the cytoplasm of animal cells has not been reported (256), neither has it been recorded in archaebacteria such as *Thermoplasma acidophilum* (547, 550). As a member of eukaryotic organisms, fungus differs from plants, animals, and bacteria. Genetically speaking, fungi are more closely related to animals than to plants. In fact, specific fungi have been seen to reduce sulfate (531), which could actually be an exception among eukaryotes in sulfate reduction.

The conservation of sulfur-reducing power down the universal phylogenetic tree invites reevaluation of our understanding of the important role of H_2S in regulating human body function. The knowledge of H_2S metabolism in bacteria, plant, invertebrate, and fungi would certainly be the asset for H_2S study on mammals.

IV. ENDOGENOUS PRODUCTION AND METABOLISM OF H_2S IN MAMMALIAN CELLS

H_2S is generated in mammalian cells via both enzymatic and nonenzymatic pathways, although the nonenzymatic pathway only accounts for a small portion of H_2S production. Among enzymes involved in H_2S production, CBS and CSE have been investigated extensively, both using pyridoxal 5′-phosphate (vitamin B_6) as a cofactor. The role of MST along with cysteine aminotransferase (CAT) in regulating endogenous H_2S level has recently been reexamined in specific types of cells and tissues. These enzymes are involved in transsulfuration and reverse transsulfuratoin pathways in different capacities and utilize specific substrates **(FIGURE 2)**. The regulation mechanisms for the expression, as well as the activities, of these H_2S-generating enzymes under physiological or pathophysiological conditions have been largely unsettled and constitute a great challenge.

A. Enzymatic Production of H_2S

1. CBS

CBS (EC 4.2.1.22) was first isolated by Braunstein et al. in 1969 under the name "serine sulfhydrase" (63). Ever since,

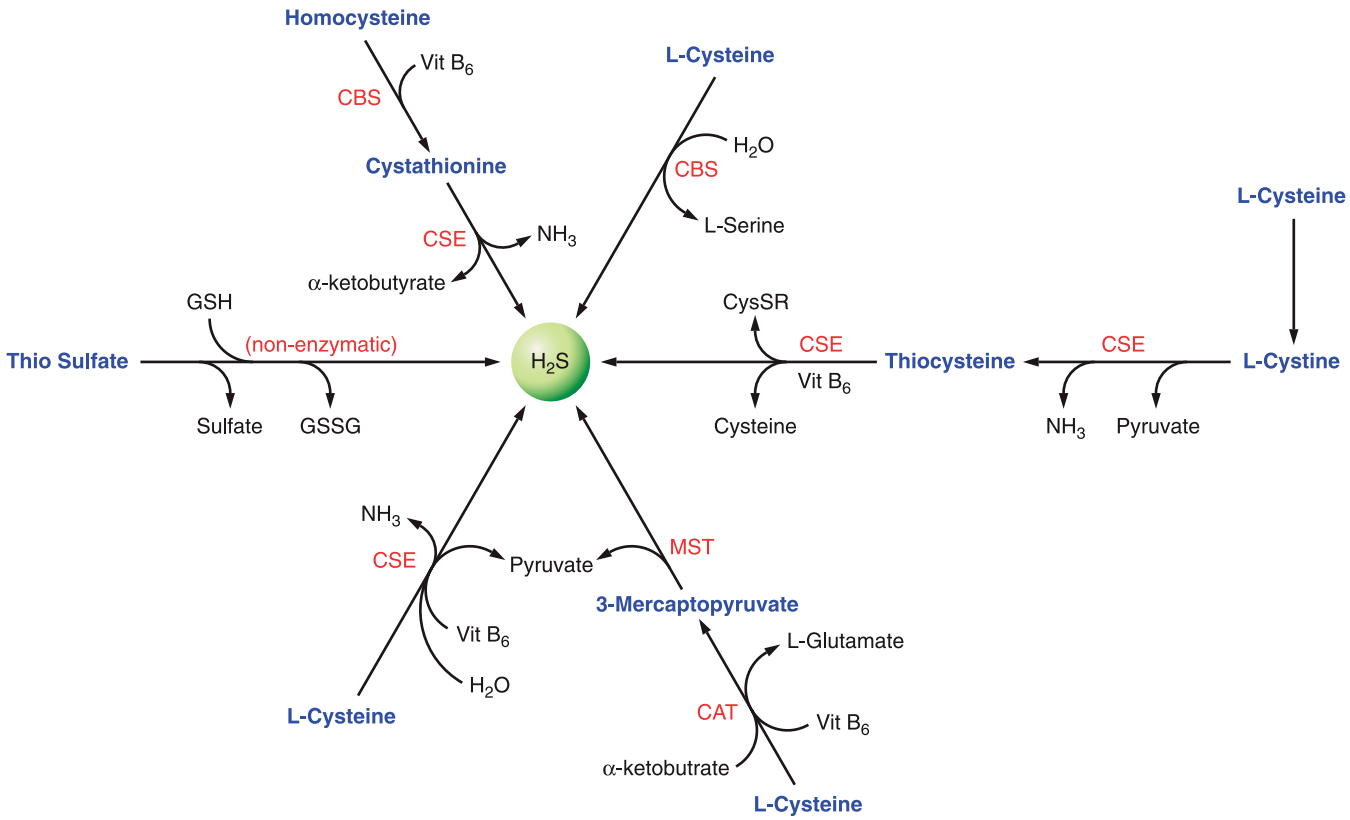


FIGURE 2. Biosynthesis and transformation of H_2S in mammalian cells. CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; CAT, cysteine aminotransferase; MST, 3-mercaptopyruvate sulfurtransferase.

different names have been given to the same protein, such as L-serine hydrolyase, beta-thionase, cysteine synthase, and methylcysteine synthase. The chromosome location of human CBS gene is on chromosome 21 (43.35–43.37 Mb), and mouse CBS gene is on chromosome 17 (31.34–31.37 Mb).

In the presence of cysteine, more so in the presence of homocysteine, CBS catalyzes the production of H_2S **(FIGURE 2)**. The most well-known reaction catalyzed by CBS is the condensation of homocysteine and serine: L-serine + L-homocysteine ⇌ L-cystathionine + H_2O.

This is the first significant step in the biosynthesis of cysteine from methionine by reverse transsulfuration. CBS also catalyzes the condensation of cysteine with homocysteine to form cystathionine and H_2S (275).

Maclean and Kraus (383) compared the catalytic efficiencies of various reactions and found that L-serine is a significantly better substrate than cysteine with homocysteine being the cosubstrate. This would underlie their observation that serine inhibits the formation of H_2S from cysteine and 2-mercaptoethanol by 50% even in the presence of a sixfold higher concentration of cysteine.

The full-length human CBS is a homotetramer consisting of 63-kDa subunits (302, 572). Each CBS subunit comprises 551 amino acid residues (302). CBS uses the cofactor pyridoxal-phosphate (PLP), which is the active form of vitamin B_6 **(FIGURE 3)**.

The NH_2 terminal of CBS contains the binding sites for both PLP and heme (protoporphyrin IX) (28). The 70-amino acid heme domain is unique for mammalian and fish CBS, not found in yeast and protozoan CBS (383). The heme in CBS is coordinated to histidine and cysteine as axial ligands in human and rodents. However, there is no clear indication for a functional role of heme in the catalytic activity of CBS. On the other hand, deletion of heme domain renders CBS insensitive to oxidative stress. As such, a redox sensor role is suggested for this heme binding domain (728). The PLP binding domain is considered to be the catalytic domain, and it is deep in the heme domain, linked by a Schiff base. Other PLP-dependent enzymes include tryptophan synthase (258), threonine deaminase, aminocyclopropane deaminase (743), succinylhomoserine (thiol)-lyase, and *O*-acetylhomoserine (thiol)-lyase (4).

The COOH terminus of CBS contains a regulatory domain of ~140 residues, playing an autoinhibitory role for the activity of full-length CBS. Binding of the allosteric activator, *S*-adenosyl-L-methionine (AdoMet or SAM), to this domain will cause a conformational change so that CBS is instantly activated. SAM is a common cosubstrate involved in methyl group transfers, transmethylation, transsulfuration, and aminopropylation pathways, and mostly produced in liver (92). Deletion of the regulatory domain con-

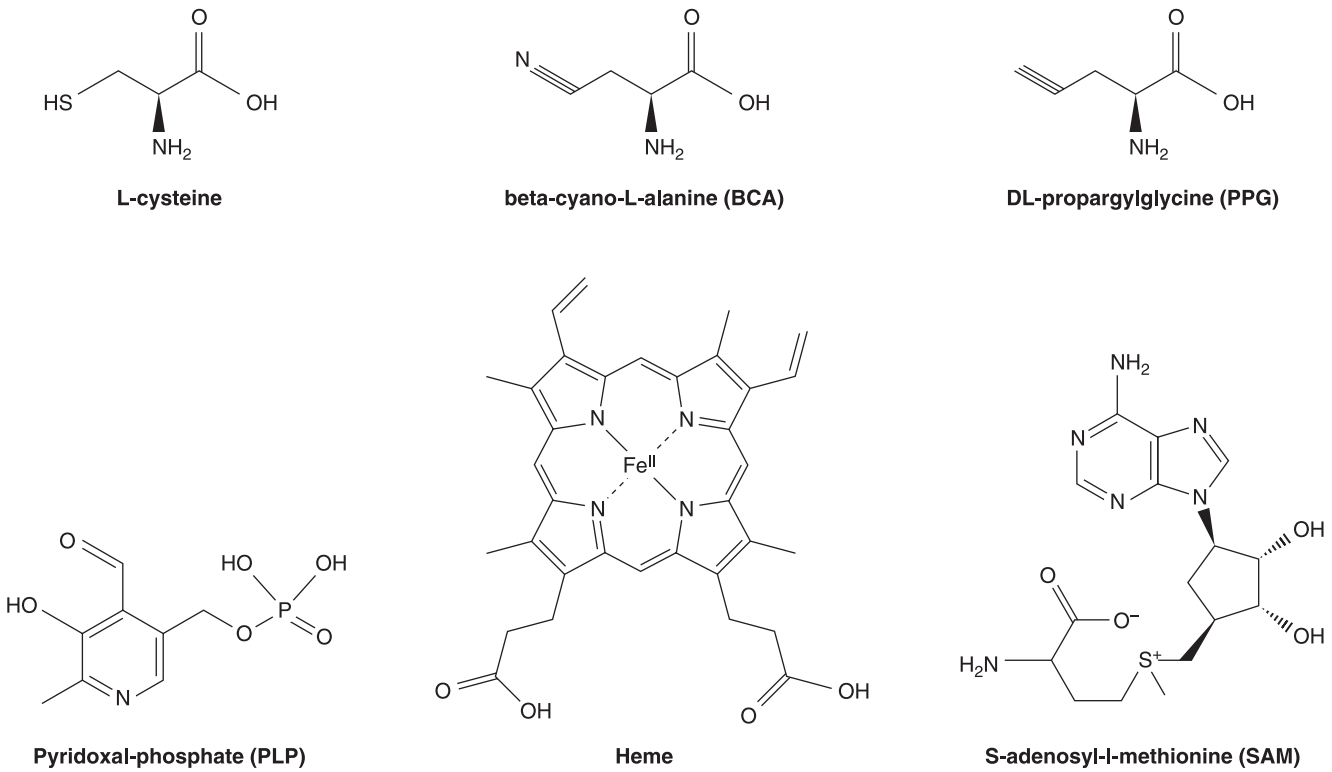


FIGURE 3. Critical molecules in the regulation of enzymatic production of endogenous H_2S.

stitutively activates CBS (598). This regulatory domain is important for maintaining the tetrameric state of the protein (28). The calmodulin binding consensus sequence (19 amino acids) has also been identified in CBS at its COOH-terminal domain. After calcium-activated calmodulin binds to this consensus sequence, the catalytic domain of CBS will be opened and the enzyme becomes active.

CBS expression is significant in the brain as the primary physiological source of H_2S in the central nervous system. Initially, CBS was found to be highly expressed in the hippocampus and cerebellum when compared with the cerebral cortex and brain stem (1). This brain distribution of CBS was later confirmed by Robert et al. using immunohistochemical technique (519). In contrast, Enokido et al. (162) argued that CBS is preferentially expressed in astrocytes rather than neurons, which is verified by combined biochemical and histological examination as well as in situ hybridization. This fits with recent findings that CBS mainly localizes to astrocytes. Another study showed that the basal H_2S level in unstimulated human astrocytes is ~3.0 μmol/g protein, which is 7.9-fold higher than in cultured microglia. More importantly, only astrocytes, not microglia, are strongly immunostained for CBS (339). Vitvitsky et al. (660) showed the incorporation of radiolabel from methionine into GSH in both cultured human astrocytes and neurons. Since the only known route for the transfer of radiolabeled methionine to GSH is via the transsulfuration pathway involving CBS and CSE, these experiments indirectly justify the existence of CBS in both astrocytes and neurons. Nevertheless, studies consistently identified the temporal expression of CBS in developing and adult mouse CNS. During the embryonic period, CBS protein level is generally low, but it dramatically increases from late prenatal to early postnatal period (162, 519).

In other tissues, such as cardiovascular system, respiratory system, testes, adrenal, and spleen from rats, mice or humans, CBS expression is rare or absent. An earlier study showed the activity of CBS, reflected by the production of cystathionine, in cultured human umbilical venous endothelial cells (677). However, in that study, no attempt was taken to detect CBS mRNA or protein in these cells, which had been cultured for 14 days with the addition of 100 μM L-homocysteine. This observation may suggest that CBS could function as an inducible H_2S generating enzyme in the vascular endothelial cells, an observation which merits further investigation. Also, the upregulation of CBS may occur when homocysteine level or other links of transsulfuration pathways are altered. As such, not only is the sulfur metabolism affected, but the endothelium-dependent production of H_2S and vasorelaxation would also be regulated under different physiological and pathophysiological conditions.

In the absence of CBS, the tissues will not be able to catabolize homocysteine via transsulfuration pathway and therefore become hypersensitive to homocysteine toxicity (275). Another consequence for this lack of CBS is that these tissues would reply on extracellular supply of cysteine due to the deficiency in cysteine synthesis.

Mutations of the regulatory domain of CBS may lead to changes in the constitutive activation of this enzyme, leading to hereditary diseases such as in homocystinuric patients (290). So far, at least 153 mutations in human CBS gene have been identified in patients with homocystinuria (324), characterized by an accumulation of homocysteine in the serum and urine. Both gain-of-function polymorphisms and loss-of-function polymorphism have been identified with the CBS gene (324).

2. CSE

Cystahionine γ-lyase (EC 4.4.1.1) has been conventionally abbreviated as CSE, CGL, or CTH. It is also known as γ-cystathionase, cysteine lyase, cysteine desulfhydrase, cystathionase, cystathioninase, cystine desulfhydrase, homoserine deaminase, or homoserine dehydratase. CSE is located on chromsome 1 (human), p31.1. There are two isoforms of human CSE. An internal deletion of 132 bp separates the longer isoform from the short one, likely the consequence of alternative splicing (355).

The CSE gene is expressed in numerous organisms including mammals, amphibian (488), and plants such as *Nicotiana tabacum* (120). Different CSE isofroms possess high sequence identity between phylogenetically distant organisms.

CSE has been described as an exclusively beta-replacing lyase with a strict specificity for the primary substrate L-cysteine and for several sulfur-containing cosubstrates (64, 429). Determination of the CSE crystal structures has recently revealed that both yeast and human CSE are virtually identical at their active sites to cystathionine γ-synthase (CGS) from *Escherichia coli*. Similar to CBS, CSE also use PLP as its prosthetic group (401).

CSE is expressed abundantly in mammalian cardiovascular system and respiratory system (245, 773). It also appears to be the main H_2S-forming enzyme in the liver, kidney, uterus, placenta, as well as pancreatic islets (735, 773). A low level of CSE is also detectable in the small intestine and stomach of rodents (180). CSE gene was initially cloned from rat liver (DDBJ/EMBL/GenBank accession no. X53460). To determine whether the same isoform of CSE is expressed in the cardiovascular system, Zhao et al. in 2001 (773) cloned and sequenced two isoforms of CSE from rat mesenteric arteries, which contained an ORF of 1197 bp, encoding a 398 amino acid peptide (GenBank AB052882). They further cloned and sequenced CSE from rat liver (GenBank AY032875) and found no differences among all the clones from artery and liver. However, these CSE clones are different from previ-

ously deposited rat liver CSE amino acid sequence (X53460) by ~5%. In earlier days, anti-CSE antibody was not available commercially. Therefore, the expression of CSE at the protein level was not determined. The first Western blot study on CSE protein expression in cardiovascular system was reported by Yang et al. in 2006 (739).

Small amounts of CSE mRNA have also been detected in the brain (165). In contrast to the liver and kidney, H_2S production in brain seems to be unrelated to CSE activity. CSE inhibitors, D,L-propargylglycine and β-cyano-L-alanine, do not suppress the production of H_2S in the brain (1), although they effectively suppress H_2S production in the liver and kidney (599).

Like CBS, CSE has been only localized in the cytosol. For example, Ogasawara et al. (449) studied the subcellular localization of CSE in the rat liver and kidney. In their study, CSE activity was mainly detected in the cytosolic fractions in the both tissues.

The second step of reverse transsulfuration is catalyzed by CSE, which is cleavage of the C-γ-S bond of cystathionine to yield L-cysteine, 2-oxobutanoate (synonyms: 2-ketobutyrate; α-ketobutyrate; α-oxobutyrate), and ammonia.

However, Steegborn et al. (594) also showed that purified recombinant human CSE cleaves cystathionine almost exclusively at the C-γ-S bond of cystathionine. As such, CSE is capable of using L-cysteine as the substrate to form two gases, H_2S and NH_3, and pyruvate **(FIGURE 2)**. The involvement of CSE in other elimination reactions includes the catalizattion of L-homoserine to form H_2O, NH_3, and 2-oxobutanoate; and that of L-cystine to form thiocysteine, pyruvate, and NH_3.

Human deficiency of CSE may lead to a metabolic disorder named cystathioninuria that is inherited in an autosomal recessive manner. The patients have an excess of cystathionine in the urine. Other diseases related to CSE mutation include hypercystathioninemia and increase the risk of developing atherosclerosis and bladder cancer (512).

3. MST and CAT

MST (or SseA) (EC 2.8.1.2) is synonymous with β-mercaptopyruvate sulfurtransferase. MST has A and B chains. The first crystal structure of MST was derived from *Escherichia coli* (583). MST is involved in cyanide detoxification as MST transfers the sulfane sulfur from substrate to cyanide ion, giving nontoxic thiocyanate and pyruvate. The cofactor of MST is zinc.

Another enzyme that has been reported to generate H_2S in rat liver is CAT (EC 2.6.1.3) (102). CAT also uses PLP as the cofactor. MST and CAT have been localized in both cytosol and mitochondria.

Working together with CAT in the presence of 2-oxoglutarate and PLP, MST may also generate H_2S (331). CAT converts cysteine to 3-mercaptopyruvate (3-MP). MST would then transfer the sulfur from 3-MP to sulfite or other sulfur acceptors or form elemental sulfur. The direct outcome of the CAT-MST pathway is the production of sulfane sulfur (or bound sulfur), not the free form of H_2S. H_2S would be consequently formed either through reduction of the atomic sulfur or released from thiosulfate or persulfides. The former requires the presence of reductants (288) and the latter, specific enzymes such as thiosulfate sulfurtransferase or thiosulfate reductase (599).

The sulfur-carrier property of MST is similar to that of rhodanese (425, 426). Furthermore, rhodanese catalyzes the production of H_2S and H_2SO_3 from the interaction of thiosulfate with glutathione. It is intriguing to ask whether rhodanese or thiosulfate sulfurtransferase or reductase can also be categorized as H_2S-generating enzymes. The crystal structures of CBS, CSE, and MST are depicted in **FIGURE 4**.

Under in vitro experimental conditions, especially with the optimal alkaline conditions (pH 9.7) and high concentration of cysteine, these reactions have been documented (599). However, under more physiologically relevant conditions with 2 mM cysteine in the reaction solution and pH at 7.4, the CAT and MST pathway failed to produce meaningful H_2S in rat liver and kidney (599). What represents a bigger challenge for the role of CAT-MST pathway is the existence of 3-MP in cells or tissues. As an unstable molecule, 3-MP is the only sulfur donor for MST, but its presence in vivo has not been directly detected to date. Suggestion has been made regarding the presence of 3-MP based on the detection of mercaptolactate-cystene disulfide, a metabolite of 3-MP, in urine (558).

In the central nervous system, MST is localized to hippocampal pyramidal neurons, cerebellar Purkinje cells, and mitral cells in the olfactory bulb in the brain (558). Immunohistochemistry and Western blot analysis reveal the presence of MST in aortic endothelium and smooth muscles and the presence of CAT in endothelium (557). In rats, MST is predominantly localized in proximal tubular epithelium of the kidney, pericentral hepatocytes in the liver, cardiomyocytes in the heart, and neuroglial cells in the brain (425).

Defects in MST in humans have been reported, but no life-threatening dysfunction is associated (128).

4. Transsulfuration and Reverse-Transsulfuration Pathways

Transsulfuration and reverse transsulfuration are two opposite processes involving the interconversion between the sulfur-containing amino acids cysteine and methionine. Reverse transsulfuration occurs in vertebrates and fungi with the final product being cysteine (182). Transsulfuration, the

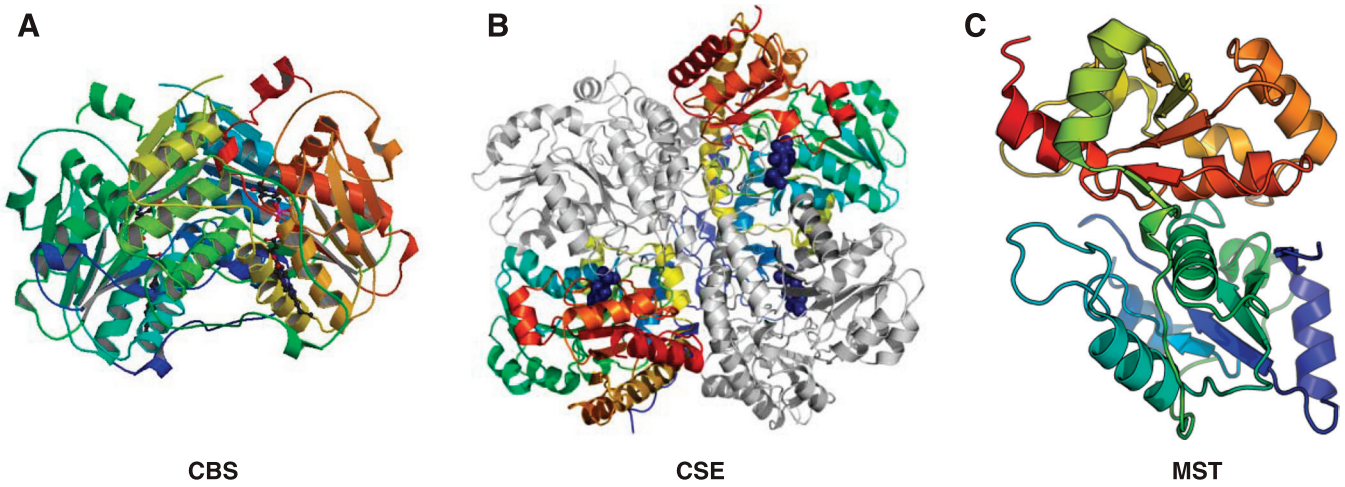


FIGURE 4. Structures of three key hydrogen sulfide-generating enzymes. *A*: structure of human cystathionine β-synthase (PDB Code: 1JBQ) (399). *B*: structure of human CSE (image by Karlberg et al., October 2006. PDB code: 2NMP). *C*: structure of human MST (image by Karlberg et al., August 2010. PDB code: 3OLH).

transformation of cysteine into homocysteine via the intermediate L-cystathionine with the final product being methioine, mainly occurred in bacteria, fungi, and plants. Transsulfuration is catalyzed by cystathionine β-lyase (CBL) and cystathionine γ-synthase (CGS) (182, 594) **(FIGURE 5)**.

A) REVERSE TRANSSULFURATION PATHWAY. In mammals, cysteine is synthesized from methionine via cystathionine by the reverse transsulfuration pathway. This pathway is believed to be the sole route for cysteine synthesis in vertebrates with CBS acting as the flux-controlling enzyme. It also functions as a catabolic pathway of methionine and its toxic intermediates including homocysteine. At elevated levels, homocysteine is an independent risk factor for cardiovascular diseases and other complex disorders. Besides homocysteine removal, reverse transsulfuration contributes significantly to the intracellular cysteine pool. Cysteine is used for biosynthesis of glutathione and is also the primary substrate for H_2S biosynthesis. The reverse transsulfuration pathway is regulated by two PLP-dependent enzymes CBS and CSE (598). CBS catalyzes the condensation of homocysteine and

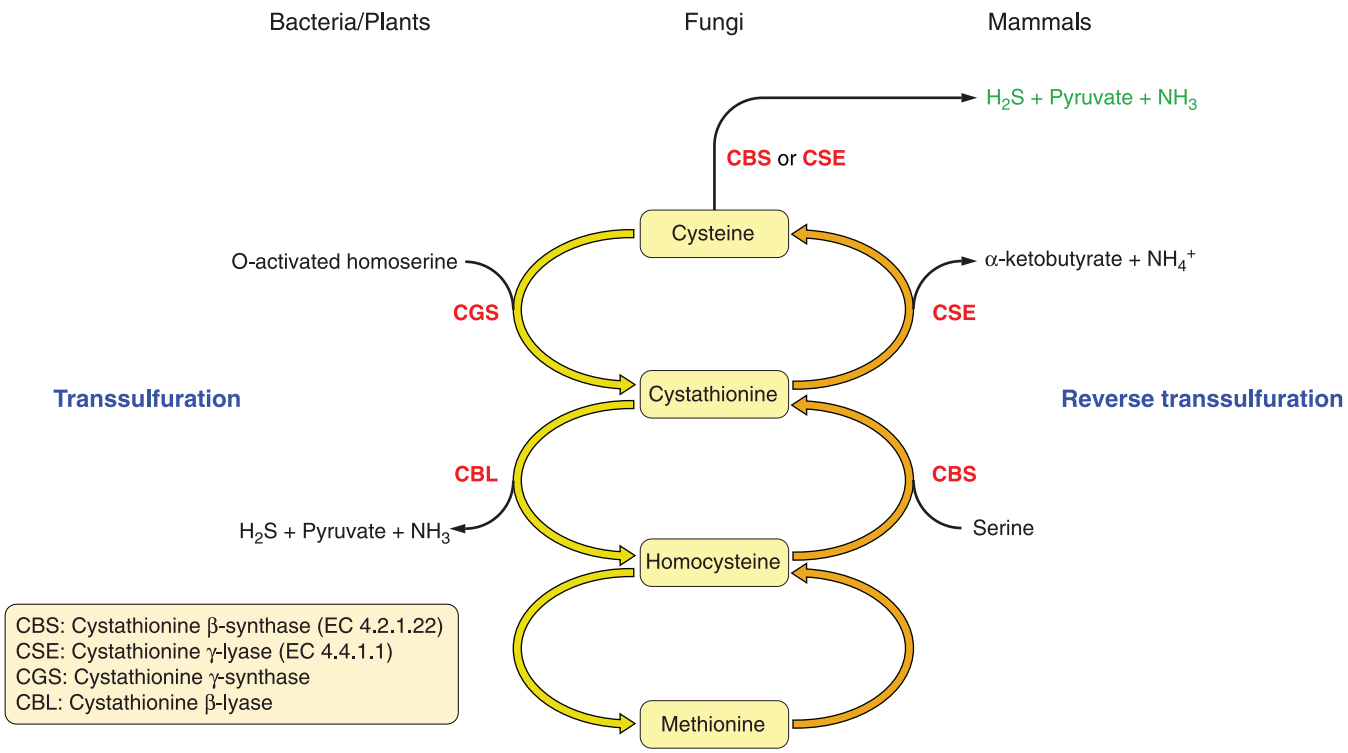


FIGURE 5. Transsulfuration and reverse transsulfuration pathways. [Modified from Steegborn et al. (594).]

serine to form cystathionine in an irreversible reaction, which explains the unidirectional flow in the reverse transsulfuration sequence from methonine to cysteine. The cystathionine is then hydrolyzed by CSE to form cysteine and α-ketobutyrate plus ammonia. The α-ketobutyrate is further catabolized by oxidative decarboxylation to propionyl-CoA, which enters the tricarboxylic acid cycle at the level of succinyl-CoA. The oxidative decarboxylation of α-ketobutyrate can be catalyzed by pyruvate and branched-chain keto acid dehydrogenase complexes. Thus the reverse transsulfuration pathway is responsible both for the catabolism of the carbon chain of methionine and for the transfer of methonine sulfur to serine to synthesize cysteine. Although all cells are capable of transmethylation and remethylation, the catabolism of homocysteine via reverse transsulfuration is restricted to certain tissues. Tissues that are not capable of conducting a sufficient reverse transsulfuration require an exogenous source of cysteine and must export homocysteine (or cystathionine) for further metabolism/removal by other tissues. Interestingly, the reverse transsulfuration pathway is also required to remove sulfur-containing amino acids under conditions of excess (28).

B) TRANSSULFURATION PATHWAY. Prokaryotes, fungi, and plants synthesize methionine from cysteine via the transsulfuration pathway employing the complementary enzymes CGS and CBL. CGS and CBL are also PLP-dependent enzymes. CBL, CGS, and CSE belong to the same PLP-γ family, but CBS is unrelated and belongs to the PLP-β family. Both CBL and CGS are homotetramers composed of ~40- to 45-kDa subunits and carry one PLP cofactor per monomer covalently bound via a Schiff base to an active site lysine.

5. Substrates of CSE-Generating Enzymes

A) L-CYSTEINE. L-Cysteine is derived from the dietary amino acid methionine. Cysteine is an α-amino acid with the chemical formula $HO_2CCH(NH_2)CH_2SH$. It is a nonessential amino acid, which means that it is biosynthesized in humans. However, deficiency in the reverse transsulfuration pahway or lack of methionine will make cysteine an essential amino acid. Alternatively, cysteine can be synthesized by the sulfurylation of *O*-acetylhomoserine to give homocysteine, which then joins reverse transsulfuration pathway to be converted to cysteine. Intracellular traffic of cysteine among different orgnalles is mediated by specific transporters (199). As the principal substrate for CSE, L-cysteine is of interest as a way of manipulating endogenous tissue concentrations of H_2S. A recent study (161) demonstrated that exogenous L-cysteine application limited the infarct size in ischemic heart. This protective effect of L-cysteine was believed to be due to the enhanced endogenous production of H_2S via CSE action. The inhibition of CSE activity with DL-propagylglycine (PPG) abolished the effect of L-cysteine.

Not all biological effects of cysteine can be explained by the production of H_2S. The side chain of cysteine is a nonpolar thiol, and often cysteine is described as a hydrophobic amino acid. Utilized as a nucleophile, the thiol side chain can participate in enzymatic reactions. The thiol is susceptible to oxidization to give the disulfide derivative cystine, which serves an important structural role in many proteins. Sulfur-containing amino acids, especially cysteine, methionine, and *S*-adenosylmethionine, are essential for the growth and activities of all cells (442). Methionine initiates the synthesis of proteins, whereas cysteine plays a critical role in the structure, stability, and catalytic function of these proteins. Cysteine is also involved in the synthesis of the major antioxidant glutathione. The correlation of cysteine supply and CSE functionality has been recently made (269, 388). CSE-KO mice fed with cysteine-limited diets exhibited decreased levels of cysteine, glutathione, and H_2S but increased plasma homocysteine level (388). These animals were tagged with the growth retardation and shortened life span. The causes of death could be the paralysis of the upper extremities and skeletal muscle atrophy after the cysteine-limiting diet (269), although in another study no paralysis phenotype was observed in CSE-KO fed with a similar cysteine-limiting diet (388). The liver function and anatomy of these animals were normal (388). In the absence of CSE expression, CSE-KO mice would be still able to produce H_2S with CBS or other putative H_2S-producing enzymes. Therefore, the lack of cysteine supply through diet might further reduce H_2S production in these animals, leading to all consequences. This hypotheis, however, is rejected because injecting the mice with NaHS daily (intraperitoneally) did not reverse or stop the cysteine-limiting diet-induced decrease growth retardation of CSE-KO mice. On the other hand, after cysteine was added to the drinking water as a supplementation to the cysteine-limiting diet, the CSE-KO mice exhibited an increase in body weight and were rescued from death. These studies suggest that CSE is critical for cysteine biosynthesis through the transsulfuration pathway, and sufficient cysteine supply is one prerequisite for animal survival in the absence of CSE.

B) HOMOCYSTEINE. Homocysteine is a sulfur-containing amino acid not found in the regular diet and primarily generated from methionine in a variety of tissues including the liver. Methionine is the precursor of SAM, a methyl donor in a number of methylation reactions involving RNA, DNA, proteins, and lipids. Homocysteine is formed upon demethylation of SAM and subsequent hydrolysis of *S*-adenosylhomocysteine and lies at the junction of two intersecting pathways. One of them converts the sulfur atom of methionine to cysteine and glutathione (reverse transsulfuration pathway). The other remethylation pathway (coupled to cobalamine, folate, and betaine metabolism) reconverts homocysteine to methionine (394). Increased plasma levels of homocysteine due to loss-of-function mutation or heterozygosity of CBS and CSE represent a well-defined risk factor

for cardiovascular, thrombotic, neurodegenerative, and pregnancy-associated diseases. Under these conditions, hyperhomocysteinemia promotes endothelial dysfunction and impairs endothelial-dependent vasodilation.

Homocysteine and cysteine are readily oxidized compared with GSH and exist largely in oxidized forms in plasma. It is clear that tissue concentrations of both homocysteine and cysteine are maintained at low levels by regulated production and efficient removal of these thiols. The body's capacity to function with low concentrations of homocysteine and cysteine is facilitated by its ability to store cysteine as GSH, which may be hydrolyzed to generate cysteine as needed; the reliance on GSH as the major cellular thiol or redox buffer; and the ability of cells to regenerate methionine (Met) from homocysteine.

6. Endogenous Inducers of H_2S-Generating Enzymes

A) CBS INDUCERS. SAM binds the COOH terminal of CBS and activates the enzyme. Eto and Kimura (169) reported that H_2S production in the brain is partly regulated by testosterone and SAM. Male brains contain more H_2S than female brains at each age, suggesting the involvement of testosterone in the regulation of the H_2S level, as may occur in the liver. The application of testosterone to female mice increases H_2S and SAM in the brain, almost reaching the levels of males. In contrast, castration of male mice decreases the levels of testosterone, H_2S, and SAM. These observations suggest that both testosterone and SAM are involved in the upregulation of CBS expression in the brain. However, this publication was retracted in 2005. It is interesting to note that the production of NO is also regulated by testosterone (568). SAM synthesis may be affected by not only testosterone, but also glucocorticoids as in the case of the liver. It has been reported that glucocorticoids stimulate CBS gene expression (507). CBS expression is also upregulated by epidermal growth factor (EGF), transforming growth factor-α (TGF-α), cAMP, and dexamethasone in reactive astrocytes (162).

B) CSE INDUCERS. NO can affect H_2S level in vascular tissues through two different mechanisms. Zhao et al. (773) for the first time discovered that NO increased CSE activity in vascular tissues. They incubated rat aortic tissue homogenate with a NO donor for 90 min, which significantly increased H_2S generation in a concentration-dependent manner. One possibility is that NO increases the activity of cGMP-dependent protein kinase, which in turn stimulates CSE. Alternatively, NO may directly act on CSE protein. Mammalian CSE protein is made up of 12 cysteines. The specific cysteine residues that interact with NO have not been determined. However, the nitrosylation of certain free -SH group of CSE in the presence of NO does represent a possibility. The second mechanism for NO-induced H_2S production is the upregulation of CSE expression. Incubating cultured vascular SMCs with a NO donor for 6 h significantly increased the expression level of CSE (773). Patel et al. (474) also found that the expression of CSE is upregulated by the NO donor, S-nitroso-*N*-acetylpenicillamine (SNAP), and CSE activity is enhanced by another NO donor, SNP (474).

CSE can be upregulated by bacterial endotoxin (270) as well as during liver regeneration (682). The apparent repression of CSE as a consequence of human immunodeficiency due to virus infection and the significant upregulation of CSE during lactation in rats have also been reported (20). CSE transcription and protein turnover rates are also affected by vitamin B_6 availability (538). Stimulation of endothelial cells with vascular endothelial growth factor (VEGF) increased H_2S release, likely due to the upregulation of CSE (467).

Testosterone is another endogenous CSE stimulator. It does not change the expression of CSE but stimulates CSE activity (75). In rat thoracic aorta preparation, testosterone increased the production of H_2S from L-cysteine, which was significantly reduced by PPG and β-cyano-L-alanine (BCA), two specific inhibitors of CSE. This would also explain testosterone-induced relaxation of rat aortic rings in vitro, which again was inhibited by PPG and BCA.

The mechanism for the transcriptional regulation of CSE is not clear, but there is evidence for the involvement of myeloid zinc finger 1 and specificity protein 1 (SP1; also known as Sp1 transcription factor) (270). Like many other transcription factors, Sp1 can bind to specific *cis*-acting sequences of the selective genes to regulate their transcription by assisting RNA polymerase binding. CSE is one of those Sp1 regulated genes. The core promotor of human CSE gene is located between −226 to +140 base pairs. There are two Sp1 consensus binding sites in the core promoter region of the hCSE gene (−164/−158 and −66/−60). The mutation of these two sites significantly decreased luciferase reporter gene activity. Overexpression of Sp1 in human aorta SMCs increased the activity of the CSE core promoter, CSE protein expression, and H_2S produciton. Mithramycin, an inhibitor of Sp1 binding, downregulated CSE mRNA expression in a dose-dependent manner. The altered interaction of Sp1 and CSE may impact on the phenotype transition during SMC differentiation. The binding of Sp1 to the human CSE promoter was increased in differentiated human aorta SMCs compared with that in the proliferative SMCs, unmasked with the chromatin immunoprecipitation assay (734). The transcriptional regulation of CSE expression by Sp1 is also demonstrated in insulin-secreting INS-1E cells. Knockdown Sp1 expression largely abolished CSE expression (766).

MicroRNAs are a class of endogenous short double-stranded noncoding RNA molecules (580). MicroRNAs targets at 3′-untranslational regions (UTRs) of mRNAs for degradation and/or translational repression in mammalian cells.

MicroRNA-21 (miR-21) is an oncogenic miRNA that inhibits apoptosis in various carcinomas and affects cell-cell proliferation and migration. The overexpression of MiR-21 was abundant in rat carotid arteries after angioplasty (276) and in proliferative human aorta SMCs as well as injured mouse carotid arteries (733). The expression of both CSE and Sp1 genes can be regulated by miR-21. Two miR-21 complementary regions in the human Sp1 3′-UTR (4917–4941 and 6449–6472) have been identified, and mutation of these two regions abolished the suppressive effect of miR-21 precursor on the luciferase reporter activity of the human Sp1 gene (733). Transfection of human aorta SMCs with miR-21 precursor for 48 h led to a dose-dependent increase in miR-21 expression and simultaneous decrease in CSE and Sp1 protein expressions. The altered CSE and Sp1 expression in this case is specific for miR-21 as miR-1 precursor treatment had no effect on CSE and Sp1 protein expressions. MiR-21 treatment also decreased H_2S production and enhanced SMC proliferation. Interestingly, miR-21 precursor transfection decreased the CSE mRNA level, but not that of Sp1. These results suggest that the downregulation of CSE mRNA expression by miR-21 is attributed to the inhibition of Sp1 protein expression by miR-21. Since Sp1 was not knocked down or inhibited in this study before the mir-21 precursor transfection, a direct effect of miR-21 on CSE expression cannot be excluded yet (733).

The expressional regulations of CSE and CBS are quite different. CSE expression in both yeast and mammals appears to be induced by oxidative stress (382), whereas transcription of the human CBS gene is clearly repressed by reactive oxygen species. The temporal and spatial expression patterns of CSE differ significantly from those of CBS during embryogenesis through to early neonatal life.

7. Endogenous Inhibitors of H_2S-Generating Enzymes

Insulin downregulates the expression of CBS in liver tissues (507). Insulin also has the potential to downregulate the expression of the CSE gene (682). Since CAT is identical to cytosolic aspartate aminotransferase (3) and mitochondrial aspartate aminotransferase (652), aspartate can competitively inhibit the production of H_2S from L-cysteine in the CAT-MST pathway (599).

B. Pharmacological Blockers of H_2S-Generating Enzymes

1. PPG

PPG (or PAG; $C_5H_7NO_2$) is an antibiotic produced by *Streptomyces sp.* (541) with a molecular weight of 113.1 **(FIGURE 3)**. PPG is membrane permeable and can be easily dissolved in organic solvents such as ethanol and DMSO at ~20 mg/ml. The solubility of PPG in physiological saline is ~10 mg/ml.

PPG irreversibly inhibits CSE through a "suicidal" inactivation. PPG has been used in different cell culture studies at concentrations of 10–20 mM, and it does not have direct cytotoxic effect at this concentration range on human aortic smooth muscle cells (735). At this concentration range, PPG significantly inhibited CSE activity and H_2S production. The IC_{50} of PPG for blocking CSE activity in rat liver preparations is 55 μM (408). PPG has been used to inject animals. Intraperitoneal injection of PPG at 30 μmol/100 g body wt inhibited CSE activity in rat liver and kidney by >95% (599). The injection also significantly suppressed H_2S production in vascular and ileum tissues (771, 773). At concentrations ranging from 25 to 100 mg/kg, PPG can reduce H_2S-associated inflammation in rodent models of pancreatitis and edema (49) as well as endotoxemia (122). Administration of PPG (3.12–75 mg/kg ip) exhibited a dose-dependent protective effect against intragastric administration of 1 ml ethanol to induce gastric injury in rats, while exogenous administration of H_2S reversed this effect (105). The level of gastric H_2S was increased after ethanol-induced gastric damage, and they were reverted by PPG. In the studies on cisplatin-induced renal damage, PPG was injected into rats twice daily with each injection at the dose of 5 mg/kg ip (189). The whole treatment lasted for 4 days. PPG reduced renal H_2S formation rate and CSE expression and inhibited cisplatin-induced tubulointerstitial lesions in the outer medulla. Increased expression of tumor necrosis factor (TNF)-α, macrophages, neutrophils, and T lymphocytes, associated with cisplatin treatment, were also reduced by PPG.

The search for true selective inhibitors for CSE has been ongoing for decades, but not much progress can be spotlighted. To date, there is no other better agent than PPG that can inhibit CSE at appropriate concentrations. Thus the availability of PPG has contributed significantly to our studies on CSE-related H_2S metabolism. The relative selectiveness and effectiveness of PPG on CSE activity are largely decided by the concentration range of this agent used (707). PPG may have nonspecific effects at high concentrations on other proteins, such as aspartate aminotransferase (619) and alanine aminotransferase (84).

2. BCA

BCA is another inhibitor of CSE. Its molecular weight is 141.1 ($C_4H_6N_2O_2$) **(FIGURE 3)**. In contrast to PPG, BCA is not readily soluble in organic solvents. Its aqueous solubility is the same as for PPG, 10 mg/ml.

BCA reversibly inhibits CSE (483). H_2S production in both rat liver and ileum tissues was inhibited in vitro by PPG and BCA in a concentration-dependent manner (773). BCA blocks H_2S synthesis activity in rat liver preparations with

an IC_{50} value of 6.5 μM and increases blood pressure in anesthetized rats induced with hemorrhagic shock by inhibiting endogenous H_2S synthesis (408). Tang et al. (620) examined the effects of endogenous H_2S on K_{ATP} currents and membrane potentials in rat mesenteric artery SMCs. They found that PPG decreased reversibly K_{ATP} currents from −212 to −125 pA, and depolarized membrane potentials from −55 to −16 mV. BCA had more potent inhibitory effect on K_{ATP} currents than PPG did (51 vs. 36%). BCA at 50 mg/kg blocked both L-cysteine- and LPS-induced hyperalgesia in rats (301). However, the nonspecific effect of BCA on other molecular targets cannot be excluded.

3. Hydroxylamine

The production of H_2S from brain homogenates is suppressed by hydroxylamine (HA) and aminooxyacetate (AOA) (307). HA is an inorganic compound (NH_2OH). It is soluble in cold water and in alcohol. HA has been used both as a reducing agent and an antioxidant. It inhibits many heme-containing enzymes. As one of those heme proteins, CBS activities can be inhibited by HA. Some non-heme molecular targets are also affected by HA (585). For example, HA may be converted to NO in vivo. It has been reported that HA or AOA had no effect on K_{ATP} currents in vascular smooth muscle cells (621).

4. AOA

AOA is also called carboxymethoxylamine, which has been used as a nonspecific inhibitor for CBS. In fact, AOA can be better used as a general inhibitor for aminotransferase (511). AOA can also form a carboxymethoxamate by trapping pyruvate to exert different biological functions (603). At 2 mM, AOA completely inhibited the sulfate and thiosulfate formation from L-cysteine and the sulfate formation from L-cysteinesulfinate in rat liver mitochondrial preparations (633). This suggests the inhibition of CAT. In this line of consideration, other inhibitors of aminotransferase may also affect endogenous H_2S level by inhibiting the activities of CBS and/or CAT. Methylglyoxal (MG) inhibits the activity of partially purified rat liver L-glutamine: D-fluctose-6-phosphate aminotransferase (ED 2.6.1.16) with an IC_{50} of ~10 μM (304). This MG effect can be reversed by cysteine (304).

5. Inhibitors of MST

As 3-MP is the substrate of MST, mercaptic acids with structural similarities with 3-MP would potentially affect MST activities. It was found that, in in vitro enzyme kinetic studies of MST, 3-mercaptopropionic acid acted as a noncompetitive inhibitor and 2-mercaptopropionic acid acted as an uncompetitive inhibitor of MST with respect to 3-MP (489).

Based on their capability of being cyanide antidotes in vivo, three α-keto acids (α-ketobutyrate, α-ketoglutarate, and pyruvate) have been tested for their effects on MST activity. All three α-keto acids were shown to be uncompetitive inhibitors of MST with respect to 3-MP. Their IC_{50} values were between 9.5 and 13.7 mM (490).

C. Nonenzymatic Production of H_2S

A minor endogenous source of H_2S is the nonenzymatic reduction of elemental sulfur to H_2S using reducing equivalents obtained from the oxidation of glucose, which has been described in erythrocytes (681). Human erythrocytes produce H_2S when provided with elemental sulfur or inorganic polysulfides (43, 548). Increased oxidative stress and hyperglycemia will promote H_2S production from this path. All essential components of this nonenzymatic path are present in vivo, including the supply of reducible sulfur. The presence of millimolar concentration of sulfur in blood circulation has been reported in humans or mice.

Sulfide, via nonenzymatic oxidation, yields thiosulfate. The latter can be converted to sulfite by thiosulfate reductase in liver, kidney, or brain tissues or by thiosulfate sulfurtransferase in the liver. H_2S can also be released from thiosulfate and persulfides. Garlic and garlic-derived organic polysulfides induce H_2S production in a thiol-dependent manner.

D. Catabolism of Endogenous H_2S

1. Expiration and Excretion

A significant amount of exhaled H_2S has been measured after intravenous administration of sodium sulfide (265). An increased amount of exhaled H_2S has also been reported after inhibition of endogenous NO synthesis. Furthermore, the endogenous polysulfide DADS increases the amount of H_2S exhaled (265). Given the fact that the endogenous production of H_2S can be altered in various pathophysiological conditions, it may be useful to evaluate the possibility of using exhaled H_2S as a diagnostic measurement. H_2S is also excreted in the urine primarily as sulfate (either free sulfate or thiosulfate) and in feces and flatus unchanged as free sulfide.

2. Oxidation

Hydrogen sulfide in mitochondria is first oxidized to thiosulfate and then to sulfite and sulfate (89). To be clear, two molecules of H_2S form one molecule of thiosulfate. Thiosulfate formation had been previously demonstrated in isolated and perfused rat livers and kidneys (30). The formation of thiosulfate is not enzymatically created, but thiosulfate conversion to sulfite and/or sulfate is catalyzed by sulfide-detoxifying enzymes. Rhodanese is reported as a sulfide-detoxifying enzyme (486). Between the two isoforms of rhodanese, thiosulfate cyanide sulfurtransferase (TST) ac-

tually can detoxify H_2S as well as thiosulfate, but MST cannot (506). The sulfite that is produced through this reaction is quickly oxidized to sulfate. Therefore, sulfate ends up being the major end-product of H_2S catabolism under physiological conditions, which is also the reason why urinary thiosulfate is determined by many to be a nonspecific marker of entire-body H_2S production. In the cecal mucosa and liver homogenates of Sprague-Dawley rats, H_2S is metabolized to thiosulfate and sulfate (354).

The oxidation of H_2S to thiosulfate in the rat liver is under the impact of heme compounds (581, 582), metal-protein complexes, and ferritin (651). The conversion of H_2S to sulfate has been observed for a long time (651, 653). The rate and the extent of sulfide oxidation were seen to be variable from one organ to another.

3. Methylation

Methylation is another catabolic pathway for H_2S. While the oxidation of H_2S occurs mainly in mitochondria, methylation mainly takes place in the cytosol. The methylation of H_2S yields methanethiol (CH_3SH). Interestingly, methanethiol is also a colorless gas with a smell like rotten cabbage. Methanethiol can be further methylated, although much more slowly, to dimethylsulfide (CH_3SCH_3), a relatively nontoxic compound. Thiol *S*-methyltransferase (TSMT) catalyzes both of these two steps.

H_2S has been reported to be methylated to methanethiol in vitro by the intestinal mucosa of Sprague-Dawley rats (697). TSMT is a ubiquitous enzyme with the highest activity in the colonic and cecal mucosa (697), but its activity has also been reported in the liver, lung, and kidney. Compared with sulfide oxidation, sulfide methylation is slow. In one study, it was shown that sulfide methylyation (697) is ~10,000 times slower than the oxidation rate of H_2S in colonic mucosa (354).

4. Scavenging

The half-life of NO in blood is counted in seconds, and it can be scavenged by oxyhemoglobin (681). H_2S seems to be more stable in protein-free solution. However, H_2S can also be scavenged by methemoglobin to form sulfhemoglobin. Therefore, the half-life of free H_2S in blood may also be short.

Yang et al. (732) reported that methemoglobin at 10 μM partly but significantly reversed the antiproliferative effect of CSE. Pretreating wild-type HEK-293 cells with methemoglobin for 1 h prior to adding 100 μM H_2S significantly abolished the antiproliferative effect of H_2S. Decreased H_2S production in CSE-overexpressed cells by methemoglobin also provided evidence that methemoglobin scavenged the endogenous H_2S. Since hemoglobin can scavenge NO, CO, and H_2S, this protein function as a common "sink" for all three known gasotransmitters.

H_2S can also be scavenged by metallo- or disulfide-containing molecules such as horseradish peroxidise, catalase, and oxidized glutathione (39, 574).

V. PHYSIOLOGICAL FUNCTIONS OF H_2S IN MAMMALIANS

A. H_2S and the Cardiovascular System

1. Chronotropic and Inotropic Effects of H_2S on Heart

The reports on the effects of H_2S on heart rates have been inconsistent. Some studies claimed a negative chronotropic effect of H_2S due to the inhibition of pacemaker cells in SA nodes. Xu et al. (726) showed that NaHS (50–200 μM) treatment of rabbits decreased the velocity of diastolic (phase 4) depolarization and rate of pacemaker firing in normal pacemaker cells in SA nodes. These negative chronotropic effects were inhibited by glibenclamide (20 μM), but not by CsCl (2 mM). The opening of K_{ATP} channels, rather than I_f channel, in pacemaker cells by H_2S is therefore likely underlies the effect of H_2S (726). The improvement of arrhythmia associated with I/R injury by NaHS has also been reported (769). Single-channel recording on single cardiac myocytes showed that NaHS at 40 μM increased the open probability of K_{ATP} channels from 0.07 to 0.15 and at 100 μM from 0.07 to 0.36 (769).

On the other hand, administration of H_2S (intravenously) at 2.8 and 14 μmol/kg did not have any effect on heart rate of rats, although the same treatment significantly lowered blood pressure of the animals (772). Administration of PPG to inhibit endogenous H_2S production also did not alter it action potentials in the pacemaker cells of rabbits (726). It is possible that at low concentration H_2S would not have significant chronotropic effect. On this note, some earlier studies using H_2S at toxic levels indeed show the impact of H_2S on heart rate.

Acute exposure of rabbits to 72–75 ppm of NaHS for 1.5 h or less caused electrocardiogramp alterations, such as cardiac arrhythmia and flattened and inverted T waves (321), although the lack of statistical analysis significantly limits the interpretation of the studies. Kohno et al. (314) reported a decrease in heart rate (10–27% of controls) in rats exposed to 75 ppm H_2S for 60 min (314). In contrast, another study found an increase in heart rates in rats exposed to 100–200 ppm H_2S for 1 h (237).

It should also be attended that baroreflex is quite different between humans and other mammals, which has to be

taken into account when evaluating the chronotropic effect of H_2S.

A negative inotropic effect means the decreased contract force or the required energy of muscular contractions, which is beneficial for lowering cardiac work load in conditions such as angina. During irreversible ischemia and reperfusion injury (I/R injury) to the isolated rat hearts, H_2S induces negative inotropic effect and reduced central venous pressure in in vitro and in vivo experiments, thus protecting the heart from I/R injury (571). A similar effect was observed in mice receiving 1 mg/kg NaHS at reperfusion in an in vivo model (277). Not all studies support a negative inotropic effect of H_2S. NaHS (50 or 100 μM) had no significant effect on the contractile status of the isolated rat ventricular myocytes in vitro (746, 747). In these isolated myocytes, a negative inotropic effect of SNP or L-arginine was observed as decreased myocyte twitch amplitudes (746) and isoproterenol (a β-adrenoceptor agonist) caused a positive inotropic effect reflected by increased twitch amplitude of ventricular myocytes (747). Surprisingly, both the negative and positive inotropic effects of NO and isoproterenol were reversed by NaHS (746, 747). The inhibition of cAMP pathway by H_2S was believed to be responsible for the reversal of the isoproterenol effect (747). Increased intracellular calcium level by NaHS after it was first lowered by NO was accounted for as the molecular mechanism for the neutralization of the contractility in the presence of both H_2S and NO (746). The physiological meaning of this intriguing role of NaHS in counteracting both positive and negative inotropic influences in the heart is not clear. The extrapolation of these observations from the isolated cardiomyocytes in vitro to the contractile dynamics of the heart in vivo under more physiological conditions should be carefully evaluated.

The opening of K_{ATP} channels in the myocardium plays a key role in H_2S-offered negative inotropic effect as glibenclamide, a classical K_{ATP} channel blocker, inhibits the cardiac effects of H_2S (206, 571). This is consistent with the negative inotropic effects of other K_{ATP} channel activators, which induce cell membrane hyperpolarization. NaHS-induced cardiac protection effect was also suppressed by 5-hydroxydecanoate (5-HD) (284). 5-HD is a mitochondrial K_{ATP} (mitoK_{ATP}) channel blocker while glibenclamide blocks the plasma membrane K_{ATP} channels. K_{ATP} activation can result in the closing of L-type calcium channels, reducing calcium entry, and inhibiting muscule contractility (606).

2. Vasorelaxant effects of H_2S

As the first identified gasotransmitter, NO relaxes smooth muscle of various vasculatures. Substances other than NO, including low-molecular-weight *S*-nitrosothiol intermediates, also contribute to the relaxation of smooth muscle (172, 424). CO has been found to dilate different vascular tissues, from conduit arteries to resistant arteries (604, 605, 716). H_2S-induced vasorelaxation has been demonstrated in numerous types of blood vessels (such as aorta, portal vein, mesenteric artery, cerebral arteries, and vas deferens) from different species (such as rats, mice, cows, guinea pigs, sheep, and humans). The EC_{50} of H_2S in inducing vasorelaxation is quite close to the reported endogenous level of H_2S in plasma, which suggests that under physiological in vivo conditions the vascular tone of resistance arteries is likely regulated by endogenous H_2S. Exogenously applied H_2S in the form of NaHS relaxed vascular smooth muscles. In addition, low concentrations of H_2S enhance smooth muscle relaxation induced by NO in the helical tissue strips of the thoracic aorta (245). Teague et al. (630) reported a summation effect between H_2S and NO on the sublimation of the twitch responses of the ileum to electrical activation. The enhancing effect of H_2S on NO-induced vasorelaxation is still controversial. Zhao et al. (773) observed that pretreatment of aortic ring preparations with H_2S inhibited the vasorelaxant effect of the NO-producing agent SNP (773). Ali et al. (5) have shown that H_2S induced vasoconstriction and increased the mean arterial pressure in rats likely by scavenging endothelial NO. It is likely that the interaction of NO and H_2S may alter the vasorelaxant properties of these two gasotransmitters. Also, the common molecular target for NO and H_2S may become desensitized after firstly encountering one of them.

The production of H_2S in the presence of NO is a different story. H_2S production by CSE in vascular tissues is increased by SNP, while the expression of CSE is upregulated by another NO-producing agent, SNAP (773). CSE contains 12 cysteine residues that are potential targets for *S*-nitrosylation. *S*-nitrosylation of CSE has the potential to increase the enzymatic activities (313).

The vasorelaxant effects of H_2S-gassed solution or NaHS solution have been studied on preconstricted vascular tissues. The potency of vasorelaxant effect of H_2S is affected by the preparations of H_2S (772). At the same concentration level, H_2S-gassed solution has much stronger vasorelaxant effects than NaHS solution does. The stimulus used to precontract vascular tissues also significantly affects the effect of H_2S. While H_2S relaxed phenylephrine- or norepinephrine-precontracted aortic tissues, high concentration of KCl (>60 mM)-induced vascular contraction was essentially not affected by H_2S. Finally, different vascular tissues manifest different sensitivities to H_2S. H_2S relaxes small mesenteric arteries much more potent than aortic tissues (112). Although rat aortic and mesenteric artery tissues produce similar levels of H_2S, H_2S is nearly sixfold more potent in relaxing rat mesenteric artery beds than relaxing rat aortic tissues. The higher sensitivity of mesenteric arteries to H_2S speaks for the importance of H_2S in regulating peripheral resistance. The mechanisms for differential vasorelaxant effects of H_2S are not clear yet, but several possibilities

exist. One explanation is the tissue-type specific distribution of the molecular targets of H_2S. For example, the expression of K_{ATP} channels possibly differs in various vascular tissues with different isoforms. The second explanation is that sensitivities of contractile proteins to H_2S and to intracellular calcium level may vary between conduit and resistant arteries (112). Also, different types of blood vessels face different sheer stress levels, possess different cellular components (smooth muscle cells, endothelial cells, and connective tissues, etc.), and have different stiffness. Finally, oxygen-dependent sensitivity of blood vessels to H_2S should also be considered. It has been reported that H_2S induced vasorelaxation at physiological O_2 levels, and this vasorelaxation occurred much faster at below physiological O_2 levels. With higher than physiological O_2 levels, H_2S has the tendency to induce vasoconstriction (313). This could result from the product of H_2S oxidation, which may mediate vasoconstriction. Blood in small peripheral vessels has lower oxygen partial pressure, and these small vessels consume oxygen at higher rate due to the high content of smooth muscle cells and low collagen. The situation is just opposite in large conduit arteries. The difference in tissue oxygen level may explain different vascular effects of H_2S. Another note worth taking is that the release of NO from *S*-nitrosoglutathione by H_2S is oxygen dependent (313).

H_2S functions as a vasodilator in cerebral circulation. Topical application of H_2S to the newborn pigs induces dilation of pial arterioles (345). This vasorelaxant effect of H_2S appears to be mediated by K_{ATP} channels as glibenclamide blocked the H_2S effect. Leffler et al. (345) further showed that L-cysteine per se dilated pial arterioles. Three lines of evidence were given to demonstrate the effect of L-cysteine was the outcome of CSE-generated H_2S. First, PPG at 10 mM blocked the vasorelaxant effect of L-cysteine, but AOA at 1 mM failed to do the same. Second, CSE proteins were detected in cerebral microvessels. While CBS proteins were detected in brain parenchyma, it was not detectable in cerebral microvessels. Third, H_2S concentration in cerebrospinal fluid was increased about fourfold after L-cysteine treatment, measured by GC-MS, which was again blocked by PPG. Whether this vasodilatory effect of H_2S is unique to newborn animal or ubiquitous to cerebral circulation at other stages of development is not known.

The involvement of various signal transduction pathways in the vascular effects of H_2S has been examined. NO and CO relax smooth muscle by activating guanylyl cyclase to increase the production of cGMP. H_2S does not affect the production of cGMP, which leads to the inference that there is a different mechanism for the effect of H_2S. Earlier studies also demonstrated that the vasorelaxant effects of H_2S on rat vascular tissues are unlikely mediated by prostaglandin, protein kinase C, or cAMP pathways (112, 772, 773). Superoxide dismutase and catalase in the bath solution also did not alter the vasorelaxant effect of H_2S, indicating that superoxide anion and hydrogen peroxide did not contribute to H_2S-induced acute vasorelaxation. Although ODQ blocked the vasorelaxation induced by SNP, it had no effect on the vasorelaxant effect of H_2S on rat aortic tissues. Therefore, under this experimental condition, the vasorelaxant effect of H_2S was not mediated by the cGMP pathway (773).

K_{ATP} channel is the major molecular target of H_2S for its vasorelaxant effect and smooth muscle hyperpolarization (771, 773). The activation of K_{ATP} channels by H_2S does not rely on the intracellular ATP level. In the ileum, glibenclamide did not interfere with the relaxation induced by H_2S (630). This finding may be seen as different membrane excitation-contraction coupling mechanisms in different muscle preparations. In effect, though H_2S inhibits KCl (20 mM)-induced contractions of aortic tissues, it does not change the contraction of ileum induced by the same concentration of KCl (630).

3. *Inhibited Vascular Smooth Muscle Cell Proliferation*

The proliferation of vascular SMCs plays a critical role in the maintenance of vascular structure and functions, and its alteration leads to vascular remodeling and various proliferative vascular diseases. However, cellular and molecular mechanisms that regulate SMC proliferation and differentiation are not fully understood. H_2S is an important endogenous modulator of cell proliferation and apoptosis (735). Serum deprivation upregulated CSE expression and H_2S production in cultured human aorta SMCs in concert with the induced SMC differentiation marker gene expressions, such as SM-MHC, calponin, and SM α-actin (733). Overexpression of CSE in human aortic SMCs inhibited cell growth and induced cell apoptosis (740). Absence of endogenous H_2S in vascular SMCs, such as those isolated from CSE gene deficient mice (KO mice), led to a significant surge in cell growth rate (737). The percentage of BrdU-positive cells in cultured SMCs and in the media of the aorta was also significantly greater in CSE KO mice than in age-matched CSE wide-type (WT) mice (737). Clearly, endogenous CSE/H_2S limits the proliferation and growth of SMCs. Furthermore, increased SMC proliferation in CSE KO mice was not secondary to the development of hypertension. The normalization of blood pressure in CSE KO mice by captopril did not reduce aortic SMC proliferation when compared with untreated age-matched CSE KO mice (737).

The endogenous level of H_2S affects the effect of exogenous H_2S on cell apoptosis. Yang et al. (740) found that NaHS induced apoptosis of human aortaic SMCs at concentrations $\geq$200 μM (740). After inhibition of endogenous H_2S production by PPG pretreatment or by knocking down endogenous CSE gene with short-interfering RNA approach, the proapoptotic effect of NaHS becomes significant at 50–100 μM. Another study reported that exogenously applied

NaHS at 100 μM inhibited proliferation and induced apoptosis of vascular SMCs from CSE KO mice, but not of SMCs from wide-type (WT) mice (737).

CSE/H_2S pathway is also involved in the development of balloon injury-induced neointima formation of rat carotid arteries. The transcriptional expression levels of CSE, CSE activity, and endogenous H_2S production were all decreased in ballon-injuried carotid arteries (400). Treatment of the rats with NaHS significantly weakened balloon injury-induced neointimal hyperplasia and reduced vascular smooth muscle cell proliferation in the lesions in vivo. Similar observations were made in the mouse where carotid artery ligation resulted in enhanced neointima formation and downregulation of CSE expression (733).

The mechanisms underlying the antiproliferative and/or proapoptotic effect of H_2S are multifaceted. One of the focal points of these studies is the involvement of the mitogen-activated protein kinase (MAPK) superfamily, including three parallel cascades which are the stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) cascade, the p38-MAPK cascade, and the classical extracellular signal-regulated kinase (ERK)/MAPK cascade. In human aortic SMCs, for example, exogenous H_2S induced apoptosis through activation of MAPK pathway. The phosphorylation of ERK transduces the apoptotic signal to its downstream enzyme cascades and eventually activates caspase-3. After the activities of ERK and caspase-3 were inhibited, the apoptosis of human aortic SMCs induced by H_2S was significantly attenuated. Therefore, the activation of ERK and its downstream factor caspase-3 likely mediates H_2S-induced cell apoptosis (735). It is worth pointing out that in many other cell types or tissues, ERK activation serves as a proliferative/antiapoptotic signal. It has been reported that the proliferation of cultured rat aortic vascular SMCs was inhibited by NaHS. At the same concentration range (50–500 μM), NaHS also inhibited ERK activity (155). Whether activation of ERK could reverse NaHS-induced proliferation inhibition was not conducted by the same researchers (155). Therefore, it is not sure whether the decreased ERK activity can account for the reported effect of NaHS on rat vascular SMCs.

In CSE overexpressed HEK-293 cells, ERK and p38 MAPK activities were significantly increased, but not in Ad-lacZ infected cells or control cells, and the cell growth was inhibited (739). The activations of ERK and p38 MAPK were also involved in H_2S-treated intestinal epithelial cells (IEC-18) (141).

The pro-apoptotic effect of H_2S may also be related to the cell cycle due to the stimulation o f cyclin-dependent kinases. *S*-diclofenac {2-[(2,6-dichlorophenyl)amino]benzene acetic acid 4-(3H-1,2,dithiol-3-thione-5-yl)phenyl ester} is a novel molecule comprising an H_2S-releasing dithiolthione moiety attached by an ester linkage to diclofenac (31). *S*-diclofenac induces a dose-dependent decrease in the survival of primary and immortalized rat aortic vascular SMCs. The cells in G_1 phase were not affected by *S*-diclofenac but asynchronized SMCs manifested with an increase in apoptotic cell death. *S*-diclofenac stabilized p53 and induced p21, p53AlP1, and Bax. But the anti-apoptotic factor Bcl-2 was not affected (31). In CSE-overexpressed cell or exogenous H_2S-treated cells, there are also an increased expression of p21Cip/WAK-1, and a downregulation of cyclin D1 (739).

The anti-proliferative and/or pro-apoptotic effect of H_2S may be of importance for the prevention of cell proliferation in disorders such as atherosclerosis, vascular graft occlusion, and neointimal hyperplasia leading to restenosis after angioplasty (687).

4. Stimulated Vascular Endothelial Cell Proliferation

The same physiological stimuli do not necessarily elicit the same functional responses from different types of cells. While H_2S inhibits vascular SMC proliferation, the gasotransmitter stimulates the proliferation and migration of vascular endothelial cells either in culture or in the whole blood vessel walls. To this end, the stimulatory effect of H_2S on ECs has been reported with cultured human umbilical vein endothelial cells (HUVECs) (88, 467) and bEnd3 microvascular endothelial cells (608). It should be noticed that the pro-proliferative effect of H_2S donors on ECs could not be detected if the concentrations of H_2S donors were higher than physiologically relevant levels. The signaling pathways underlying the stimulatory effect of H_2S on EC proliferation are complex and inconclusive. The stimulation of PI-3K/Akt pathway, K_{ATP} channels, and MAPK and the inhibition of sGC/cGMP pathway by H_2S have all been suggested in ECs (611). Increased intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured human saphenous vein endothelial cells by NaHS treatment has also been reported (34). This increase in $[Ca^{2+}]_i$ was mostly due to calcium release from ryanodine receptor-coupled endoplasmic reticulum and due to capacitative Ca^{2+} entry to a smaller extent. To date, there is no report to link the effect of H_2S on $[Ca^{2+}]_i$ levels in ECs to H_2S-stimulated EC proliferation (34).

H_2S also protects ECs from different stress damages. Hyperglycemia decreased the viability of ECs by increasing oxidative stress and nuclear DNA injury. This hyperglycemia stress results in impaired endothelium-dependent vasorelaxation. In cultured microvascular ECs, the hyperglycemia-induced EC damage was suppressed by supplementation of exogenous H_2S to the culture media. CSE overexpression increased EC viability by 6% compared with the native ECs, facing the same hyperglycemic culture conditions. On the other hand, knocking down the expression of endogenous CSE with siRNA deteriorated hypergly-

cemia-enhanced oxidative stress in ECs (608). Extending their observations from cultured endothelial cells, Suzuki et al. (608) overexpressed CSE gene in thoracic aortic rings isolated from Sprague-Dawley rats. This in vitro transfection reserved the endothelium-dependent vasorelaxant properties of the vascular rings in the presence of hyperglycemia (608). The important role of CSE/H_2S in protecting ECs from hyperglycemic damage was further demonstrated in CSE KO mice. The isolated thoracic aorta rings from CSE KO mice were manifested with much more severely damaged endothelium-dependent relaxations than that from WT mice when incubated with the same hyperglycemic conditions in vitro (608).

For more focused review on the role of H_2S in angiogenesis, readers are referred to section VIII.

5. Is H_2S an EDRF or EDHF?

An earlier study claimed that the relaxation of rat aortic tissues induced by NaHS was not altered by the removal of the endothelium (245). Unfortunately, the concentrations of NaHS used to study the endothelium-dependent vasorelaxation were not specified in this study. Zhao et al. (773) reported that the endothelium dependency of the vasorelaxant effect of H_2S was closely related to the concentrations of H_2S. The removal of the endothelium limited the relaxation of rat aortic tissues induced by H_2S at a single dose, but the maximum relaxation induced by H_2S at concentrations $\geq$1 mM was independent of endothelium. The absence of endothelium shifted the H_2S concentration-response curve to the right with the IC_{50} changed from 136 to 273 μM. The expression of CSE protein was shown in endothelial layer of mouse vascular tissues (738). The activation of muscarinic cholinergic receptor increases intracellular calcium level in endothelial cells, and the calcium-calmodulin complex activates CSE and produces H_2S. Several lines of evidence support this conclusion. *1*) H_2S formation by endothelial cells was markedly augmented by the calcium ionophore A23187, but inhibited by the calcium chelator BAPTA or the calmodulin antagonist W-7. *2*) Methacholine treatment of endothelial cells caused a triple increase in H_2S level. *3*) Blockade of cholinergic receptor with atropine abolished methacholine-stimulated H_2S production. *4*) The recombinant CSE directly bound to calmodulin. This binding was diminished in the presence of either the calcium chelator EGTA or W-7. *5*) Methacholine-induced endothelium-dependent relaxation of resistance mesenteric arteries was significantly diminished in CSE deficient mice (738). Yang et al. (738) also showed that calcium or calmodulin alone did not alter the catalytic activity of the purified CSE, but a twofold increase in the enzyme activity was achieved with the presence of both calcium and calmodulin.

Another potential source of endothelium-generated H_2S is MST and CAT-catalyzed reactions, the two enzymes being located in aorta endothelium and using cysteine and α-ketoglutarate as substrates (557). Once released from the endothelium, H_2S relaxes beneath the vascular smooth muscles (738). This mode of action of H_2S is very familiar to that of NO. Considering that eliminating or limiting nitric oxide synthase (NOS) activity in various vascular beds does not completely remove endothelium-dependent vasorelaxation, additional EDRF must fill the gap. Correspondingly, it has been proposed that H_2S is an EDRF for small resistance arteries and that NO is an EDRF for large arteries (682, 684). Future studies are expected to examine NO-mediated endothelium-dependent relaxation of large arteries from CSE knockout mice so that the relative contribution of NO and H_2S to endothelium-dependent vasorelaxation can be deciphered.

The endothelium-derived hyperpolarizing factor (EDHF) is one of EDRFs. What makes EDHF a unique property, different from other putative EDRFs, is its specific action of hyperpolarizing vascular SMCs and then being able to close voltage-dependent calcium channels. The vasorelaxant effect of EDHF is mainly mediated by small-conductance K_{Ca} channels and aided by intermediate-conductance K_{Ca} channels, which can be blocked by the coapplication of apamin and charybdotoxin. It is still up for debate as to whether EDHF is a diffusible EDRF or a simple electronic phenomenon that relays membrane potential change in endothelium to the underneath SMCs through low-electrical resistance myoendothelial gap junctions (684). K^+ ions, endothelium-derived C-type natriuretic peptide, H_2O_2, and *P*-450 metabolites/epoxyeicosatrienoic acids have been proposed as diffusible candidates for EDHF.

H_2S possesses many common features of an EDHF. The coapplication of apamin (50 nM) and charybdotoxin (50 nM) significantly weakened H_2S-induced relaxation of endothelium-intact rat aortic tissues (773). The removal of endothelium similarly reduced H_2S effect as apamin/charybdotoxin did. This infers that the vasorelaxant effect of H_2S is related to the generation of EDHF. The endothelium-dependent vasorelaxing effect of H_2S becomes even more significant in small resistance arteries, such as isolated and perfused rat mesenteric artery bed (MAB). The removal of endothelium significantly reduced the H_2S-induced dilation of MAB by approximately sevenfold. The EC_{50} of H_2S-induced blood vessel dilation changed from 22 μM in the presence of endothelium to 161 μM in the absence of endothelium (112). This tissue type selective endothelium-dependent effect of H_2S is similar to that of EDHF. The smaller the size of the arteries (such as mesenteric and coronary arteries), the greater the contribution of EDHF to the endothelium-dependent vasorelaxation (193). As such, EDHF would play a minor role in regulating the tone of conduit arteries, but a major role the coronary, mesenteric, and carotid arteries.

H_2S also exerts a long-term effect on the proliferation of vascular endothelial cells as aforementioned. In whole animal experiments, it was shown that NaHS injection (10 and 15 μmol·kg^{-1}·day^{-1} ip) significantly promoted neovascularization in mice (679).

H_2S might be released from vascular SMCs and then stimulate endothelium of small peripheral resistant arteries. Consequently, EDHF may be released from endothelial cells or H_2S be released from vascular endothelium to act as the very EDHF. This hypothesis still waits to be ratified. The fact is that H_2S can be produced both in vascular SMCs and endothelial cells. Yang et al. (738) demonstrated that CSE is expressed more abundantly in endothelial cells than in vascular SMCs in mice, and knocking out CSE gene in mice significantly reduced endogenous H_2S level in the vascular tissues. Conservatively speaking, H_2S is a creditable candidate for EDRF, and it also shares common features with the putative EDHF. The targets of H_2S are located on both vascular SMCs and endothelial cells. One target on vascular SMCs is K_{ATP} channels, and another one is charybdotoxin/apamin-sensitive K_{Ca} channels in vascular endothelial cells. The latter is the target of EDHF. Activation of these two types of channels by H_2S jointly hyperpolarizes smooth muscle cells, leading to vasorelaxation.

A significant advance in exploring the role of H_2S as an EDHF was made recently. Mustafa et al. (421) showed that H_2S indeed hyperpolarized the isolated rat mesenteric arteries, whereas the NO donor SNP failed to do so, detected with a voltage-sensitive fluorescent dye (DiBAC) (421). Compared with the mesenteric artery tissues from WT mice, the same tissues from CSE KO mice had much lower hyperpolarization response to acetylcholine stimulation. Acetylcholine-induced membrane hyperpolarization of the isolated aortic ECs from WT mice was also significantly greater than that from CSE-KO mice. Direct application of NaHS to the culture medium also hyperpolarized the cultured human aortic endothelial cells. Furthermore, H_2S induced *S*-sulfhydration of Kir6.1 subunit of K_{ATP} channels heterologously expressed in HEK-293 cells and IK_{Ca} channels in primary human aortic endothelial cells (421), detected by a modified biotin switch assay. It is suggested that these ion channel sulfhydration could be the molecular mechanism underlying H_2S-induced vascular hyperpolarization.

Although promising, the role of H_2S as an EDHF cannot be firmly determined without several critical lines of evidence. Direct recording of H_2S-induced membrane potential change in vascular SMCs in the presence of endothelial cells has not been conducted, which is needed to qualify H_2S as an EDHF. Whether endothelium produced H_2S hyperpolarizes ECs as well as SMCs and whether the presence of endothelial cells is the prerequisite for H_2S-induced SMC hyperpolarization have been unknown. H_2O_2 has been argued to be an EDHF (395, 749). H_2S has significant effect on H_2O_2 metabolism. Low level of NaHS has been shown to decrease the production of H_2O_2, $ONOO^-$, and O^{2-} in the presence of homocysteine, and improved cell viability in cultured rat aortic A-10 cells (400). Whether the endothelium-dependent vasorelaxant effect of H_2S is mediated by altered H_2O_2 production or altered redox status in endothelial cells is still not clear. EDHF has been noted as having a greater role to play in regulating endothelium-dependent vasorelaxation in female mice, whereas NO and PGI_2 are the predominant EDRFs in male mice (684). To date, whether gender heterogeneity affects the role of H_2S as an EDRF has been unclear.

6. Vascular Effects of H_2S in Nonmammalian Vertebrates

Some studies have addressed the action of H_2S on nonmammalian vertebrates, and phylogenetic surveys of H_2S responses among the nonmammalian vertebrates have been provided (151, 152, 452). These studies demonstrated that nonmammalian vertebrates exhibit complex patterns of vascular responses to H_2S, ranging from relaxation to constriction to triphasic response (151). This complexity is linked to the blood oxygen level, the unique relationship of H_2S with other gasotransmitters, and a wide spectrum of signaling pathways involved from K_{ATP} channels to calcium homeostasis in these animals (452, 455). The shared profiles of endogenous production of H_2S and various vasoactive reactions to H_2S between mammalian and nonmammalian vascular system further speak of an ancestral gasotransmitter role of H_2S in cardiovascular system.

7. Effects of H_2S on Muscle Contractility in Invertebrates

There has been little information about potential signaling actions of H_2S in invertebrate tissues. *Urechis caupo* (Echiuridae) is a large (60 g or more) marine echiuran worm found along the California coast. *U. caupo* body wall tissue produced H_2S in the presence of L-cysteine and PLP (285, 286). NaHS at 0.01 mM had no effect on the tension development of *U. caupo* body wall circular muscle strips. At 0.01 and 1 mM, NaHS caused a small but significant contraction of the muscle strips. SNP at 0.01–1 mM had no effect on the muscle contractility. However, the combined application of NaHS and SNP produced the contractile reponse much greater than NaHS alone (286). At concentrations above 5 mM, NaHS inhibited body wall muscle contraction in *U. caupo,* but this effect is largely toxic in nature. H_2S at 0.05 to 0.1 mM also impairs or prevents associative learning and long-term memory in the freshwater snail *Lymnaea* (526), but whether this affected muscle tone was not tested. NaHS alone had no effect on the contractility of brachial muscles of the clam *M. mercenaria*, but it potentiated the contractile action of 5-hydroxytryptamine (198).

This is similar to the action of NO on 5-hydroxytryptamine-induced contractions of the same muscle. The activation of soluble guanylate cyclase (sGC) seems responsible for the effect of NaHS. These studies, although limited in the number of invertebrate species or tissue types, nevertheless indicate that H_2S may act as an endogenous signaling transmitter in invertebrates, and that H_2S, whether by itself or in combination with other signaling molecules, has the capacity to stimulate muscle contraction, rather than H_2S-induced muscle relaxation in mammals. It is also important to note that both *U. caupo* and *M. mercenaria* are found in habitats with high levels of environmental H_2S, and indeed multiple sulfide-detoxification mechanisms have been described in *U. caupo* (13, 285) and other sulfide-adapted animals (661, 662). Whether other invertebrates respond in a similar way to endogenous H_2S as these sulfide-adapted invertebrates remains to be seen.

B. H_2S and the Nervous System

In mammalian central nervous system (CNS), CBS was found highly expressed in the hippocampus and the cerebellum (307, 308). CBS is mainly localised to astrocytes (162, 261) and microglial cells (248). Immunohistological study showed that CBS is expressed both in brain neurons and astrocytes (345). Additionally, in the adult brain CBS is highly expressed in some neurons, such as Purkinje cell neurons and hippocampal neurons (519). The neuronal expression and localization of MST have been summarized in section IV. The CBS-based production of H_2S involves the activation of Ca^{2+}/calmodulin pathway after neuronal excitation.

H_2S exerts multifaceted and important effects in CNS through modulation of neurotransmission and neuromodulation. Glutamate is an important excitatory amino acid that functions as a neurotransmitter. Glutamate in mammalian brain is best known for its role in learning and memory, such as induction of long-term potentiated potential (LTP) and perception of pain. LTP is a memory consolidation process, which is ignited by a brief period of high-frequency presynaptic stimulation (5–100 Hz), and this initial stimulation would enhance the postsynaptic response to subsequent presynaptic stimulation for many hours/days after the high-frequency tetanus. Abnormal glutamate metabolism can lead to excitatory neuronal injury. The neurological effects of glutamate are mediated by *N*-methyl-D-aspartate (NMDA) receptors in both central and peripheral nervous systems, with exceptions in the bone and pancreatic islet (144). To date, a direct agonist role of H_2S on NMDA receptors is unknown, but it has been found that at physiologically relevant concentrations H_2S selectively enhances NMDA receptor-mediated currents and expedites the induction of hippocampal LTP in rats (1, 307). On the other hand, high concentration of H_2S would damage brain and lead to decreased learning and memory function. Repeated exposures of rats to H_2S (125 ppm) over a period of 5 wk did not alter the animals' memory on a previously learned spatial task (470). The acquisition of a new spatial task by the animals was largely not affected by H_2S daily treatment. However, these animals showed impaired ability in finding all of the reinforcers prior to the end of a trial. Moreover, a new reversed contingency maze task demonstrated that H_2S-treated animals made more overall arm entries than controls with a 16-arm radial arm maze, showing an impaired learning ability.

NMDA receptors expressed in *Xenopus* oocyte are modulated by H_2S, and the stimulation of NMDA receptors by H_2S was abolished after the inhibition of adenylyl cyclase-specific inhibitor MDL-12,330A (89). It is thus reasoned that H_2S may stimulate the production of cAMP, which then activates protein kinase A (PKA). The NMDA receptor subunits possess specific phosphorylation sites for the action of PKA. The consequent phosphorylation of NMDA receptor subunits results in the activation of NMDA receptor-mediated excitatory postsynaptic currents. H_2S-induced increase in cAMP production and activation of PKA had also been previously reported in primary cultures of brain cellsas well as neuronal and glial cell lines.

Not only activating NMDA receptors, H_2S also directly increases glutamate secretion. Garcia-Bereguiain et al. (202) applied three to six pulses of 250 μM NaHS with 10-min intervals per pulse to rat brain tissue. With this relatively high level of NaHS stimulation, extracellular concentrations of glutamate were increased from physiological concentrations of 2–5 to 10–15 μM and cell death occurred. H_2S-induced cell neuron death was abolished by NMDA blocker MK-801 and glutamate antagonist DL-2-amino-5-phosphonovaleric acid. Considering the concentrations of NaHS and its application frequency, the increased glutamate release and cell death may reflect a toxicological situation (202).

The interaction of H_2S and NMDA receptors has the potential to affect other neuronal activities, such as epilepsy, neuropathic pain, stroke, and Parkinson's diseases. Prolonged activation of NMDA receptors causes calcium overload in cells and eventually leads to cell death. It has been reported that the blockade of NMDA receptors inhibits H_2S-induced cell death in neurons (113). H_2S may promote excitation and regulate survival/death decisions of neurons. NMDA receptors have important roles in conditions such as stroke, neuropathic pain, epilepsy, and Parkinson's disease (144).

Another example of H_2S effect on neurotransmitter receptors is GABA. GABA is a major inhibitory neurotransmitter, serving 20–30% of all synapses in CNS (291). Deficiency in GABAergic inhibition leads to febrile seizures and neuronal hyperexcitability (107, 227). H_2S has been shown

to decrease hippocampal damage induced by recurrent febrile seizures by enhancing GABAergic inhibition (227). Rather than increasing GABA level, H_2S in fact upregulates $GABA_B$ receptors at both mRNA and protein levels located at pre- and postsynaptic sites (227). This upregulation of receptor expression is likely associated with an increases in $[Ca^{2+}]_i$, which would stimulate Ca^{2+}-dependent transcription (21, 117). The increased GABAergic inhibition by H_2S may find its application in other situations where the excitation/inhibition balance is disturbed in CNS, such as seizures and epilepsy (227), stimuli leading to pain (301), and cerebral ischemia (502).

Glial cells possess neurotransmitter receptors and respond to transmitters (125). Reciprocal interactions between neurons and glia coordinate neuronal functions. Glia communicate with each other and with neurons by propagating their signals as Ca^{2+} waves (469). The Ca^{2+} waves often appear to be initiated at sites of contact with neurons, suggesting that the glial Ca^{2+} waves are initiated by neuronal excitation (101). It has been proposed that the reciprocal interactions between neurons and astrocytes are mediated by H_2S, resulting in the regulation of synaptic activity. Astrocytes, a type of glia, respond to a neurotransmitter released from neurons. In primarily cultured astrocytes or hippocampal slices, NaHS elicits a Ca^{2+} influx and a minor intracellular calcium release (427). This effect of NaHS was mimicked by neuronal excitation. As the Ca^{2+} waves in astrocytes can only be observed in the presence of neurons and tetrodotoxin (TTX) suppresses the induction, the effect of H_2S is believed to be due to Ca^{2+} wave propagation from neurons to astrocytes and among neighboring astrocytes (427).

The effects of H_2S on catecholaminergic and amino acid neurotransmission have been noticed. Sublethal or lethal concentrations of H_2S inhibit monoamine oxidase and result in an increase in norepinephrine (NE) and epinephrine contents in the hippocampus, striatum, and brain stem, but not in the cortex and cerebellum (693). Kulkarni et al. (329) reported that NaHS or Na_2S inhibited the release of [^{3}H]NE from isolated and superfused porcine iris-ciliary body, which was triggered by electrical field stimulation. The researchers also found that the inhibitory effects of H_2S donors on NE release were attenuated by AOA and PPG, which is quite puzzling since AOA and PPG inhibit CBS and CSE to decrease endogenous H_2S level. It is not clear how the decreased endogenous H_2S level would inhibit the effects of exogenous H_2S. Furthermore, NaHS had no effect on basal release of NE (329). Some insights on the role of H_2S in catecholamine metabolism have been revealed in rainbow trout chromaffin cells. H_2S stimulates catecholamine secretion via membrane depolarization followed by Ca^{2+}-mediated exocytosis, which was not altered by the nicotinic receptor blocker hexamethonium (481). H_2S increased intracellular calcium in cultured cerebellar granule neurons (CGN) (202). Chronic exposure to low concentrations of H_2S increases serotonin and NE concentrations in the cerebellum and frontal cortex of neonatal rats (529, 573).

In recent years, evidence for the role of H_2S in the regulation of hypothalamo-pituitary system has been obtained. KCl-evoked release of corticotrophin-releasing hormone (CRH) from rat hypothalamic explants was inhibited by NaHS in a concentration-dependent manner (140). The application of SAM mimicked the effects of NaHS and inhibited stress-related glucocorticoid increase. However, NaHS or SMA treatment did not affect the release of CRH under resting conditions (140). Moreover, both exogenous H_2S donors (NaHS and Na_2S) and endogenous H_2S inhibited the release of potassium-evoked D-[^{3}H]aspartate from isolated porcine and bovine retinae (459).

Suppression of oxidative stress is another action H_2S takes in the brain. H_2S inhibited HOCl-mediated inactivation of α_1-antiproteinase and protein oxidation, HOCl-induced cytotoxicity, intracellular protein oxidation, and lipid peroxidation (701). H_2S also protects brain endothelial cells against methionine-induced oxidative stress (648).

C. Pain Control

Both pronociceptive and antinociceptive effects of H_2S have been reported. As generally observed, the activation of T-type Ca^{2+} channels may underlie the pronociceptive effect of H_2S, and the activation of K_{ATP} channels may explain the antinociceptive effect. The use of different preclinical models of pain may also cause the inconsistence of H_2S effects. In a rat model of visceral pain induced by colorectal distension, H_2S induced antinociceptive effect on the perception of painful sensation (145). The transactivation of mu opioid receptor (MOR), but not kappa and delta receptors, appears required for H_2S-induced analgesia as the central administration of CTAP (a MOR antagonist) and MOR antisense inhibited H_2S-induced analgesia. The MOR involvement in H_2S-induced analgesia was further confirmed with the neuronal-like cells SKNMCs. In these cultured cells, H_2S also transactivated MOR and caused their internalization, which was inhibited by LY294002 [a phosphatidylinositol 3-kinase (PI3K) inhibitor], and glibenclamide (a K_{ATP} channels blocker).

The administration routes of H_2S would be another explanation for different roles of H_2S in pain control. Intracolonic application of NaHS increased visceral nociceptive behavior in mice that was accompanied by referred abdominal hyperalgesia and allodynia (396). However, visceral sensitivity was not affected when NaHS was delivered intraperitoneally rather than into the colon. Mibefradil, an inhibitor of T-type Ca^{2+} channels, blocked the nociceptive effect of NaHS. Endogenous H_2S plays a pro-nociceptive

role in a mouse pain model with LPS-induced mechanical paw hypernociception, but it may not directly act on nociceptive neurons (129). This conclusion is based on two lines of evidence. First, the pretreatment of mice with PPG to inhibit CSE and endogenous H_2S production reduced the hypernociception. Neutrophil recruitment to the plantar tissue was also prevented by PPG treatment. Second, LPS-induced production of the hypernociceptive cytokines, TNF-α, IL-1β, and CXCL1/KC was not altered by PPG. PPG also had no effect on hypernociception induced by PGE_2, a directly acting hypernociceptive mediator. H_2S also offers nociception in reducing pain associated with pancreatitis via targeting at T-type Ca^{2+} channels (441).

Quite opposite to a pro-nociceptive role of endogenous H_2S, exogenous NaHS plays an antinociceptive role once administered to the mice systemically by inhibiting both LPS- and PGE_2-induced mechanical hypernociception. In this regard, exogenous H_2S functions as a direct hypernociceptive factor with its target on nociceptive neurons. Glibenclamide abolished the antinociceptive effect of NaHS against PGE_2 induced hypernociception, thus supporting the involvement of K_{ATP} channels in nociceptor sensitization. It should be noticed that although NaHS was administered systemically, only peripheral antinorciception was manifested since the thermal nociceptive threshold in the hot-plate test was not altered by the same NaHS treatment.

H_2S in the enteric nervous system offers anti-inflammatory, spasmolytic, and prosecretory actions. Schicho et al. (542) showed the coexpression of CSE and CBS in more than 90% of guinea pig and human submucous and myenteric neurons. Only CSE was detected, however, in myenteric interstitial cells of Cajal (542). NaHS increased spike discharge in 23% of guinea pig and 36% of human submucous neurons, but had no effect on Ca^{2+} mobilization in cultured guinea pig enteric neurons. The excitatory effect of NaHS was reduced significantly by capsaicin desensitization and capsazepine, but not by glibenclamide. These results suggest the activation of transient receptor potentials vanilloid receptor 1 (TRPV1) receptors on extrinsic afferent terminals, which in turn activates enteric neurons, and K_{ATP} channels are not the target of NaHS in this action. NaHS (0.2–2.5 mM) concentration-dependently increased chloride secretion from human and guinea pig submucosa/mucosa preparations, but not in the colonic epithelial cell line T84. The secretory response was reduced significantly by TTX (0.5 μM), capsaicin (10 μM), and the TRPV1 antagonist capsazepine (10 μM). As such, the effect of NaHS may be mediated by an action on extrinsic afferents expressing TRPV1. However, the inhibition of intestinal motility by H_2S was similar in WT and TRPV1 knockout mice (200). Capsazepine and other selective TRPV1 antagonists (AMG9801, SB705498, and BCTC), LY294002 (PI3K inhibitor), SKF96365 (store operated calcium channel blocker), 2-APB (inositol triphosphate blocker), and atropine reduced NaHS-evoked mucosal secretion in guinea pig and human colon (323). These observations suggest that there may be multiple targets for H_2S in the gastrointestinal (GI) tract, and TRPV1 is only partially responsible. The role of endogenous H_2S in this mucosal secretion was indicated as L-cysteine application induced secretion that was diminished significantly by capsaicin desensitization, by the CBS inhibitor AOA, and by the CSE inhibitor PPG (200). NaHS also increased capsazepine- and LY294002-sensitive spike activity in afferent neurons of guinea pig, which was mediated by neurokinin receptors (323). H_2S acts on TRPV1-expressing afferent neurons of which the activity is regulated by PI3K pathway and calcium levels. The increased afferent neuronal activities result in local release of substance P and the consequent activation of cholinergic secretomotor neurons. GI mucosal secretion is finally triggered.

D. H_2S and the Endocrine System

One of the mostly studied endocrine organs for H_2S metabolism is the pancreas. CSE appears to be the major H_2S-generating enzyme in pancreatic islet due to its protein expression abundance, the observation that CSE knockout significantly reduces H_2S production from islets, and the fact that PPG abolished the most H_2S production from cultured INS-1E cells (741, 754). The expression of CBS has also been shown in rat pancreatic tissues or cloned rat pancreatic beta-cell line. In mouse pancreases, the expression of CBS mRNA was reported, but whether CBS is located to pancreatic islets or other parts of the pancreas has been unclear (754). Another study in 2007 examined the expression of CBS in HIT-T15 cells, an insulinoma pancreatic beta-cell line derived from Syrian hamster. Their data did not show any CBS proteins in native HIT-T15 cells (6). In contrast, the expression of CBS protein in mouse pancreatic islets and a mouse beta-cell line MIN6 (294) was reported. CBS expression was also found in pancreatic acinar cells (617).

Insulin release from pancreatic islets is a critical event in homeostatic control of glucose metabolism and in pathogenic process of insulin resistance development, including diabetes. Exogenously applied H_2S at physiologically relevant concentrations significantly inhibits high glucose-induced insulin release from INS-1E cells. The effect of endogenous H_2S on insulin release has been also demonstrated as adenovirus-mediated overexpression of CSE in INS-1E cells reduces insulin release but knockdown of CSE expression with siRNA or the application of PPG increases it (741). Furthermore, L-cysteine inhibits insulin release from the isolated mouse pancreatic islets (294).

H_2S-induced insulin release inhibition is largely related to the stimulation of K_{ATP} channels in beta cells. This has been shown in INS-1E cells from a pancreatic insulinoma cell line (741) and in another insulin-secreting cell line, HIT-T15

(6). Exogenously applied H_2S had no effect on the whole cell K_{ATP} currents in INS-1E cells until its concentration reached 300 μM. However, in the presence of PPG that blocks the endogenous H_2S production, K_{ATP} channels were activated by H_2S at concentrations equal to or lower than 100 μM (741). These results suggest that K_{ATP} channels in INS-1E cells are already fully stimulated by endogenous H_2S, thus maintaining the basal channel activity. After knocking down CSE gene expression in INS-1E cells, endogenous H_2S level was significantly lowered and the K_{ATP} channels are reconditioned to react with additional H_2S stimulation. The opening of K_{ATP} channels leads to membrane hyperpolarization. With this condition, insulin release from pancreatic islets would be inhibited because of reduced Ca^{2+} influx.

INS-1E cells suffer from apoptosis with decreased cell viability when CSE is overexpressed or when the cells are exposed to exogenously applied H_2S (740). Both exogenous H_2S (100 μM) and Ad-CSE transfection inhibited extracellular signal-regulated kinase1/2 (ERK1/2) but activated p38 MAPK. The pro-apoptosis effect of H_2S on INS-1E cells was due to elevated endoplasmic reticulum (ER) stress via p38 MAPK activation (740). In conclusion, H_2S is an important endogenous regulator of pancreatic structure and functions. By inducing cell apoptosis, H_2S may reduce pancreatic β cell mass, leading to reduced insulin production. By activating K_{ATP} channels, H_2S may inhibit pancreatic insulin release. These effects of pancreatic H_2S, individually and collectively, may represent a novel mechanism for homeostatic control of pancreatic insulin metabolism as well as glucose homeostatic regulation. It becomes no surprise that altered H_2S metabolism in pancreas is closely linked to the pathogenesis of diabetes, a topic that will be reviewed in detail in section VIII.

E. H_2S and the Immune System and Inflammation

Among many controversial areas in H_2S study, the role of H_2S in inflammatory processes is certainly a case in point. H_2S has been reported to exert both pro-inflammatory (122, 360, 618, 758, 762–765) and anti-inflammatory effects (160, 166, 248, 365, 571, 755). The upregulation of CSE and the consequent increase in H_2S production induced by LPS or pro-inflammatory cytokines, for example, can be viewed as a pro-inflammatory action (360, 427) or as an anti-inflammatory reaction as a compensatory protection mechanism. The controversial views on the role of H_2S in inflammation cannot be readily explained by the amounts of H_2S generated or the inflammation models tested.

1. *Anti-inflammatory Effect of H_2S*

It has been known long ago that H_2S possessed an antiproliferative effect on T lymphocytes (655) and induced apoptotic death of polymorphonuclear cells (391). These effects would limit the development of inflammation. A recent study showed that injection of rats with H_2S donors, NaHS and Na_2S, inhibited leukocyte infiltration and adherence to vascular endothelium, and edema formation in a hindpaw edema model induced by the air pouch and carrageenan (755). And vice versa, inhibitors of H_2S synthesis increase leukocyte adhesion, leukocyte infiltration, and edema formation (755). These effects of H_2S were seen irrespective of the inflammatory stimuli used (carrageenan, aspirin, or fMLP). Injection of mice with a single Na_2S bolus also significantly increased animal survival rates, which suffered from acute lung injury caused by combined burn and smoke inhalation. The inflammatory lungs saw decreased tissue IL-1β levels and increased IL-10 levels, and attenuated protein oxidation after Na_2S injection (166). In cultured human skin keratinocytes (HaCaT cells), NaHS application significantly reduced $CoCl_2$-induced cell injuries and inflammatory responses. Increased GSH level and decreased ROS generation in the presence od NaHS were coupled with reduced secretions of IL-1β, IL-6, and IL-8 (731).

Neuroinflammation is a case in point for elucidating the role of H_2S in inflammatory process. The activation of glial cells and release of inflammatory factors within brain, together with the recruitment of peripheral immune cells, jointly result in neuroinflammatory damage. This damage will deteriorate or lead to neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (524). In primary cultured microglia and astrocytes from human and rats, or murine immortalized BV2 microglial cells, NaHS attenuates LPS-induced production and release of NO and TNF-α (248).

NaHS administration prevented the inflammation-associated reduction of gastric mucosal blood flow and, importantly, reduced acetyl salycilic acid (ASA)-induced leukocyte adherence in mesenteric venules (359). Inhibited production of pro-inflammatory factors and enhanced production of anti-inflammatory cytokines or proteins are the other mechanisms proposed for the anti-inflammatory effects of H_2S. Hu et al. (246) found that the conditioned media from rotenone (10 nM)-treated microglia significantly decreased the viability of SH-SY5Y neuronal cells. However, this effect was alleviated in the neuronal cells treated with the conditioned media from NaHS plus rotenone cotreated microglia. At low concentrations, rotenone fails to decrease cell viability of SH-SY5Y cells, but it is enough to stimulate microglia activation. Hence, the observed protective effect of H_2S against rotenone, at least in part, arises from the suppression of pro-inflammatory factors released by rotenone-induced microglia.

The anti-inflammatory effect of H_2S is also realized by the upregulation of anti-inflammatory and cytoprotective

genes, such as heme oxygenase (HO). H_2S-induced upregulation of HO expression in pulmonary smooth muscle cells (451) and macrophages (501) has been shown, which is believed as the consequence of ERK activation (533). Upregulation of HO would yield more CO and bilirubin that oxidative stress and inflammation will be counteracted.

Nonsteroidal anti-inflammatory drugs (NSAIDs) significantly reduced gastric H_2S formation by downregulating CSE expression and activity in isolated and perfused normal rat liver (180). The suppressed anti-inflammatory role of H_2S by NSAIDs may contribute to the increase in leukocyte adherence, which then casues gastric injury. In this regard, H_2S has been shown to prevent the adherence of leukocytes to the vascular endothelium and decreased gastric mucosal blood flow induced by aspirin treatment (180). Administration of H_2S also prevented many of the "pro-inflammatory" effects of NSAIDs, including the elevation of intracellar adhesion molecule (ICAM)-1 and Lymphocyte function-associated antigen-1 (LFA-1) expression and the increase in mucosal TNF-α expression (180).

2. Pro-inflammatory Effect of H_2S

Experimental evidence has been presented to show H_2S as a pro-inflammatory factor in various animal models, including hindpaw edema (50), acute pancreatitis (49), LPS-induced endotoxemia (360), and cecal ligation and puncture-induced sepsis (765). Septic shock is an acute systemic inflammation with massively increased NO formation and elevated levels of inflammatory factors, including cytokines. Septic shock damages vascular endothelium and impairs tissue respiration, and is associated with high mortality in humans. It has previously been reported that excessive activation of K_{ATP} channels occurs in LPS-induced hypotension and hyporeactivity to vasoconstrictor agents (203). As H_2S is an identified endogenous opener of K_{ATP} channels, it was speculated that abnormal synthesis or activity of H_2S may play a part in septic or endotoxic shock. Indeed, tissue H_2S formation was increased in a septic shock rat model induced by cecal ligation and puncture (255). Intriguingly, plasma H_2S concentrations were also markedly increased in patients with septic shock. LPS injection increased plasma H_2S concentration and augmented liver and kidney CSE activity in mice (359).

The aforementioned studies suggest that increased endogenous H_2S could be harmful to the inflammatory tissues or organs. If this were the case, inhibiting the activity of H_2S-generating enzymes would be beneficiary. To support this view, Collin et al. (122) showed that pretreating animals with PPG markedly inhibited LPS-induced rise in lung and liver myeloperoxidase (MPO) activity and ameliorated lung and liver tissue damage. PPG treatment also blunted the increase in the pro-inflammatory cytokine and chemokine levels, and abolished leukocyte activation and trafficking in LPS-induced endotoxemia. In cecal ligation and puncture-induced sepsis, PPG treatment attenuated inflammatory response by reducing neutrophil infiltration and reducing animal mortality. On the other hand, NaHS treatment of these animals significantly aggravated septic inflammatory damages (758, 763–765). H_2S has also been reported to stimulate the generation of pro-inflammatory cytokines from human monocytes (776).

What are the mechanisms for the pro-inflammatory action of H_2S? One of such schemes is that H_2S stimulates sensory nerve endings, releasing endogenous tachykinins such as substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A, and thereby contributing to neurogenic inflammation. More than two decades ago, studies in the rat showed the activation of sensory nerves after inhalation of H_2S at toxic levels (496). Recent studies reported that relatively high concentrations of NaHS release both SP and CGRP from guinea pig airway slices in vitro (647). Cerulin-induced pancreatitis was deteriorated by H_2S through SP-neurokinin-1 receptor related pathway in mouse pancreatic acinar cells. TRPV1 antagonist capsazepine inhibited the release of SP and CGRP. In addition, NaHS contracted guinea pig bronchial and tracheal strips, which was reversed by capsaicin, and intratracheal instillation of NaHS in anesthetised guinea pigs caused bronchoconstriction and extensive airways protein extravasation (369). Thus it seems likely that at least part of the effect of inhaled H_2S is mediated by activating sensory nerves and eliciting a neurogenic inflammatory response. Whether endogenously derived H_2S in the lung plays a similar role is yet to be determined, but it is of interest that H_2S produces similar TRPV1-dependent contractile effects on the rat urinary bladder (472). Furthermore, activation of TRPV-1 has been reported to mediate neurogenic inflammation in caerulein-evoked pancreatitis (257). In this respect, enhanced plasma H_2S levels have recently been demonstrated in this condition. Once again, administration of PPG reduced the pancreatic inflammation and partially reversed the lung inflammation associated with this condition.

3. Factors Defining the Identity of H_2S in Inflammation

The fluctuation of endogenous H_2S level is an impossible parameter, with which one determines whether increased H_2S metabolism is anti-inflammatory or pro-inflammatory. A recent clinical study reveals increased H_2S production in the synovial fluids of the joints of patients as the consequence of the inflammatory induction of H_2S synthesizing. H_2S levels in synovial fluids from 20 rheumatoid arthritis patients (62.4 μM) were significantly higher than those from osteoarthritis patients (25.1 μM, $n = 4$). The elevation of H_2S level in the joint fluids is correlated well with clinical scores of inflammation and rheumatoid arthritis (703). Would this increase in H_2S production be a limiting mea-

sure or a pathogenic step for arthritis development? In a 1939 study, the indication was given that rheumatoid arthritis patients had lower sulfur metabolism and the supplementation of elemental sulfur might be therapeutically in favor (123). But no measurement of H_2S metabolism in the affected joints was undertaken. In short, one would be cautious to link the changes in H_2S metabolism to its role in inflammatory alterations simply based on clinical association analysis.

A number of factors are involved in determining whether H_2S is anti-inflammatory or pro-inflammatory. The concentrations and administration routes for H_2S may yield different inflammatory outcomes. The median sulfide lethal dose in rats has been described to be ~3 mg/kg intravenously (610). However, a wide range of H_2S concentrations has been employed in inflammation studies from 0.05 to 5 mg/kg (561). It has been argued that at low concentration H_2S is anti-inflammatory and at high concentration, pro-inflammatory, but this has not always been the case. Administration routes for H_2S may also impact the outcomes. Some studies used continuous intravenous infusion of H_2S, while others used a bolus administration. Even at the same concentration, H_2S may cause the opposite effects depending on its releasing rate. The slow releasing of H_2S from a novel H_2S donor GYY4137 inhibited LPS-induced release of pro-inflammatory mediators and increased the synthesis of the anti-inflammatory chemokine IL-10. In contrast, the fast release of H_2S from NaHS increased the synthesis of proinflammatory factors (706).

Other factors influence the role of H_2S in inflammation include animal species (rats vs. mice and others), inflammation models (regional vs. systemic inflammation), and the organ origin of H_2S (brain vs. pancreas, etc.). Finally, the role of endogenous H_2S compared with exogenous H_2S should be carefully evaluated. Most studies to date have used exogenously applied H_2S donors to show the role of H_2S in inflammation. This approach may be useful in determining the therapeutic value of H_2S in inflammation but would not substantiate the role of endogenous H_2S in the process. Use of PPG in inflammation study is valuable for determining the role of endogenous H_2S, but its value is limited. Direct and solid evidence should be derived from inflammation models in the animal that lack the expression of CSE or CBS or both. Feeding heterozygous CBS knockout mice with a homocysteine-rich diet leads to increased leukocyte adherence, increased P-selectin expression, and increased vascular permeability in the brain (293). This observation would partially portray an anti-inflammatory role of endogenous H_2S in neurogenic inflammation. No inflammation studies have been conducted on CSE KO mice. Furthermore, it is worth to noticing that the genetically engineered animals may have other phenotypes that would alter the animals' inflammatory reactions, not directly related to H_2S metabolism.

E. H_2S and the Respiratory System

1. *Distribution of H_2S-Catalyzing Enzymes Along Airway*

CSE protein has been detected in the airway and vascular SMCs in rat peripheral lung tissues using immunohistochemical staining (109). Therefore, endogenously generated H_2S may participate in the regulation of contractility of airway smooth muscles. Li et al. (364) detected CBS mRNA in rat lung tissues, but no attempt to detect CBS protein in that study was taken. The expression of both CBS and CSE in human airway smooth muscles, on the other hand, has been shown by Western blot analysis (534). The lung tissues from cow and sea lion also express CBS, CSE, and MST as detected with one-dimensional Western blot analysis and immunohistochemistry (457).

2. *Respiratory responses to exogenous or endogenous H_2S*

The major environmental exposure to H_2S in humans is through the respiratory tract. Acute human exposure to a low level ($\leq$50 ppm) of H_2S results in ocular and respiratory mucous membrane irritation. Nasal congestion and pulmonary edema in some cases ensue (509). On the other hand, human volunteers who were exposed to low concentration of H_2S (e.g., 5 ppm) for 16–30 min did not change their ventilation mechanics, although their maximum oxygen uptake has been increased (46). Even for 15 min inhalation of 10 ppm H_2S, pulmonary function tests in both males and female human subject did not reveal abnormalities (47). Another study followed a group of 10 patients with asthma who were exposed to 2 ppm of H_2S for 30 min. No significant changes in airway resistance or specific airway conductance were reported (272). In animal experiments, acute H_2S inhalation protects the lung from ventilator-induced lung injury (VILI). Mechanical ventilation of mice for 6 h (tidal volume 12 ml/kg) with room air or synthesized air significantly damaged the lung as reflected by inflammatory changes, edema formation, apoptosis, upregulation of the stress proteins such as HO-1 and heat shock protein 70, and VILI score. Inclusion of 80 ppm in the ventilation air offered anti-inflammatory and anti-apoptotic protection and prevented VILI (170).

The respiratory consequences of chronic or long-term human exposure to low levels of H_2S have been investigated mostly based on epidemiological data (33, 272). Many of these studies were not closely controlled or followed up so that the exposure levels of H_2S prior to the work could not be determined or distinguished from other environmental pollutants. Some studies showed symptoms related to bronchial hyperresponsiveness or the refractiveness of the lung to H_2S (346). One Canadian study surveyed 175 oil and gas workers who had been exposed to the sour gas (236). Thir-

ty-four percent of the workers showed no decrease in spirometric values, while the eight percent of workers who had lost consciousness experienced shortness of breath with physical activity, wheezing, and tightness in the chest.

The nasal epithelium is among the first groups of cells in the respiratory system to come in contact with ambient H_2S in the respiratory system. The nasal respiratory epithelium is heterologous in cell compostion, including squamous, respiratory, transitional, and olfactory epithelium. They are sensitive to H_2S stimulation and possess the capacity to regenerate and self-repair in response to external stimuli, including H_2S. Chronic nasal exposure to 200 ppm H_2S in rats altered the expression profiles of multiple genes in nasal epithelial cells (520), including those involved in cell cycle regulation, protein kinase regulation, and cytoskeletal organization and biogenesis, but that of cytochrome oxidase gene expression was not affected. Damage of other upper respiratory tract epithelium by high concentration of H_2S has also been reported (127). These direct H_2S actions can be extrapolated to other types of lung tissues and cells. However, some studies showed that intraperitoneal injection of NaHS affected neuronal control of breathing via altering neurotransmitter metabolism in the brain stem (317). In fact, NaHS induced apnea more effectively if injected into animals peripherally (8). These results suggest that neuronal transmission between lung and brain is also responsible for respiratory responses to H_2S. At the molecular level, H_2S targets at multiple enzymes to alter their functions. Cytochrome *c* oxidase in mitochondrial respiration chain is one of those enzymes sensitive to H_2S (482). Inhibition of this enzyme by H_2S may disrupt the electron transport chain and jeopardize cellular energy generation. A severe intoxication by H_2S would lead to central respiratory arrest.

3. Airway Restriction and Relaxation

Kubo et al. (326) investigated the effects of NaHS on the contractility of isolated mouse and guinea pig bronchial rings. It was found that NaHS at 0.01–10 mM had no effect on the basal tension of the tissues without precontraction. After being precontracted with carbachol, the mouse tissue rings were significantly relaxed by NaHS at 0.1–3 mM, but the guinea pig tissue rings showed only a marginal relaxation. The mechanism for NaHS-induced bronchial relaxation in the mouse has been unclear. Blockade of K_{ATP} channels, soluble guanylyl cyclase, cyclooxygenase (COX)-1 or COX-2, or tachykinin receptors did not alter the effect of NaHS (326). On the other hand, inhibition of voltage-dependent calcium entry in airway SMCs by H_2S may explain H_2S-induced airway smooth muscle relaxation. Ryu et al. (534) found that ACh-preconstricted muscle strips were relaxed by NaHS. This muscle relaxant effect of NaHS was explained by the decrease in intracellular free calcium in fura 2-loaded human airway SMCs by NaHS (50–250 μM). Interestingly, Na_2S (100 μM to 1 mM) had no effect on $[Ca^{2+}]_i$. KCl (60 mM)-induced elevation of $[Ca^{2+}]_i$ was also abolished in the presence of NaHS or Na_2S (50 μM-3 mM).

In addition to its relaxant effect on airway muscles, H_2S relaxes vascular tissues in the lung (243). NaHS treatment significantly decreased the mean pulmonary artery pressure in Wistar rats under hypoxia. The proliferation of pulmonary artery SMCs in pulmonary artery wall was also inhibited by NaHS treatment. This anti-proliferative effect of NaHS could be lined to the reduced expression of collagen I and III, elastin, and TGF-β3 protein in pulmonary arteries of rats under hypoxia.

4. Pulmonary Collagen Metabolism

Endogenous H_2S level in the lungs is important in limiting collagen accumulation in the wall of pulmonary small artery. Chronic hypoxia challenge of male Wistar rats increased mean pulmonary artery pressure and decreased plasma level of H_2S. The expression of collagen type I and III in small pulmonary arteries was also increased. NaHS treatment of hypoxic rats (intraperitoneally) reversed these abnormalities (757). The inhibition of abnormal accumulation of collage type I and III in the wall of small pulmonary arteries by H_2S is not only found under hypoxic conditions. The same role of H_2S on collagen remodeling was also revealed in aortocaval shunting model of Sprague-Dawley rats with high pulmonary blood flow (367, 368).

G. H_2S and the Reproductive System

Ambient H_2S at occupational relevant concentrations appears to be benign for reproductive activity in animal experiments. Male and female Sprague-Dawley rats were exposed to inhalation of H_2S (10–80 ppm) daily (6 h/day) continuously before and after breeding (154). H_2S inhalation did not change the production of normal and motile sperm or reproductive organ tissue weight of male F_0 rats. For female F_0 rats, H_2S inhalation did not change the success rate or process of pregnancy and litter size. The pups who inhaled H_2S between postnatal day 5 and 18 exhibited normal growth and development pattern.

Earlier studies already showed a trend that exposure of pregnant Sprague-Dawley rats to 28–110 mg H_2S/m^3 for 7 h/day on gestation day 6 until day 21 postpartum might increase mean parturition time and prolonged labor (232). However, due to the lack of strict statistical analysis, no conclusion could be reached. Sidhu et al. (562) previously showed that L-cysteine and NaHS relaxed pregnant rat uterus in vitro, although there was no information given about nonpregnant uterus relaxation.

The aforementioned studies focused on the effects of exogenous H_2S on mammalian reproductive systems, but the

physiological role of endogenous H_2S in this regard has not been fully investigated. CBS and CSE have been located in the pregnant and nonpregnant uterus, in fetal membranes and placenta of the rat, and in human placenta (474). These two enzymes are also expressed in female genital tissues, such as the clitoral and vaginal smooth muscles from New Zealand white rabbits (589). Intrauterine tissues in the pregnant rat and the human placenta produce measurable amount of H_2S (474). In the clitoral and vaginal smooth muscles from rabbits, the endogenous production of H_2S was confirmed, although it is also significantly lower than that in liver tissues (589). In the rat, H_2S production rate in uterus, fetal membranes, and placenta follows the order from greatest to weakest. L-Cysteine, NO donor SNP, or low oxygen level significantly increased H_2S production in intrauterine tissues. These observations suggest that H_2S could induce vasorelaxation in the placenta in response to the NO and changed oxygen level, which is important for the maintenance of uterine quiescence during pregnancy.

The metabolism of homocysteine is closely regulated by CBS. Deficiency in CBS expression results in hyperhomocysteinemia, which affects female reproductive function in many aspects. Early pregnancy loss, congenital birth defects, and maternal obstetric complications such as preeclampsia are some of the related abnormalities. In this regard, the infertility of CBS homozygous knockout mice can be informative. These mice have abnormal estrus cycle and increased progesterone response during pseudopregnancy induction. Their ovaries and ovulated oocytes appeared to be normal, but placental and uterine masses were decreased at day 18 of pregnancy and morphologically changed. The pregnant mice had normal number of uterine implantation sites but with a low number of surviving embryos. After CBS-deficient ovaries were transplanted to normal ovarectomized recipients, fertility was restored. These observations led the researchers to believe that uterine failure is the major cause for the infertility of CBS knockout mice (224). Hyperhomocysteinemia or other factor(s) in the uterine environment associated with CBS knockout mice would be the cause for the dysfunctional uterine. Studies using CSE knockout mice, on the other hand, showed a normal fertility of female mice, and these mice have significant homocysteinemia (738). Therefore, the plasma homocysteine level may not be a determining factor for uterine handling of embryos. Would the altered endogenous H_2S level in uterine matter? It may be so should uterine tissues mainly use CBS as the major H_2S-catalyzing enzyme. In this case, eliminating CBS, but not CSE, would significantly reduce local H_2S production, left a malfunctioned uterine. This scenario needs to be determined.

Expressions of CBS and CSE in human penile tissues have been detected with the similar abundant mRNA levels. Immunohistochemical staining revealed the existence of CSE only in peripheral nerves of human corpus cavernosum, but both CSE and CBS are stained in muscular trabeculae and penile artery smooth muscles (136). The basal level of H_2S production in human corpus cavernosum is relatively low, but in the presence of 10 mM L-cysteine, it is significantly increased by severalfold, which was partially inhibited by PPG and/or AOA (136).

The powerful vasorelaxant effect of H_2S on the penile artery suggests an important physiological role of H_2S in the erectile response of human corpus cavernosum. di Villa Bianca et al. (136) showed that NaHS (0.1–1 mM) relaxed the preconstricted human corpus cavernosum strips in vitro, independent of the presence of endothelium or the function of endothelial NOS (eNOS). The relaxant effect of NaHS depends on the nature of the stimuli that were used to preconstrict the tissue strips. The strongest relaxant effect of NaHS manifested itself when U46619 or endothelin-1 (modulators of the Rho kinase pathway) was used to precontact the tissues, followed by phenylephrine. The activation of K_{ATP} channels in corpus cavernosum strips is believed to underlie the tissue relaxation, since glibenclamide or high concentration of KCl blocked NaHS effect. The role of endogenous H_2S in erectile response was referred based on two sets of experiments. In experiment 1, application of L-cysteine to the isolated corpus cavernosum strips caused tissue relaxation, which was inhibited by AOA. In experiment 2, intracavernous administration of L-cysteine to the rat significantly increased intracavernous pressure, which was inhibited by intravenous administration of PPG. These experiments indicate the role played by endogenous H_2S in erectile response. It is a pity that the relative contribution of CSE and CBS to the H_2S effect is not dissected out as in in vitro experiment 1 in which only CBS activity is manipulated and in in vivo experiment 2 in which only CSE activity is blocked.

The female equivalent animal model of male erectile response has been used to study the existence and mechanism of H_2S pathway in female sexual physiology (589). Vaginal and clitoral cavernosal smooth muscle strips were isolated from New Zealand white rabbits. These tissues were relaxed by NaHS in a concentration-dependent manner. The inhibition of cAMP, NO/cGMP, and K_{ATP} channels partially and respectively inhibited NaHS effects. Compared with the potency of nitroglycerine and sildenafil, the relaxant potency of NaHS is significantly lower (589). These pilot studies indicate a potential role of H_2S in modulating female sexual responses.

H. H_2S and the GI System1.

Bacterial Production of H_2S in GI System

Endogenous H_2S level in GI system is made up of two components. The first one is sulfate-reducing bacteria present in the lumen of the large intestine. The second

one is from mammalian cells in the GI tract, which will be discussed later. H_2S can be produced at 0.2–3.4 mM in the GI tract of mice and humans by the intestinal microbiota from alimentary, such as the enterobacterial flora, which use endogenous sulfur-containing compounds including amino acids. At this high concentration range, H_2S highly likely interacts with other compounds and molecules and alters the response of colonic epithelium cells to such compounds, such as those from the absorption of phytochemical constituents including isothiocyanates, flavonoids, and carotenoids. At excessive concentrations, H_2S is known to severely inhibit cytochrome *c* oxidase, the terminal oxidase of the mitochondrial electron transport chain, and thus mitochondrial oxygen consumption. However, the concentration of free (unbound) sulfide may be much lower due to the capacity of fecal components to bind the sulfide (55).

H_2S is mainly metabolized by the colonic mucosa to thiosulfate and sulfate. This oxidation process in colonic epithelial cells involves the activities of sulfide quinone oxidoreductase, sulfur dioxygenase, and rhodanese. The functional significance of a powerful sulfide oxidation in intestinal epithelium would be the protection of GI system against high local concentrations of H_2S and the protection of other remote tissues from the potential damage induced by the overflow of H_2S (195).

Local high concentration of H_2S in GI system may have physiological importance. Certainly, GI tract cells have become adaptive to this environment. Pretreatment of human colonic epithelial cells with 1 mM NaHS increased lactate release, decreased cellular oxygen consumption, and decreased cell proliferation. Decreased activities of cytochrome *c* oxidase subunits I and II and uncoupling of respiratory chain were also seen with 1 mM NaHS, which was similar to the effect of hypoxia. A proportional slowdown in all cell cycle phases induced by NaHS explains these adaptive responses (351). Thus low concentration of H_2S is able to increase the cell respiration and to energize mitochondria, allowing these cells to detoxify and to recover energy from luminal sulfide. Clinical and animal studies indicate that H_2S in the colonic mucosa may be pro-inflammatory in most case, but have anti-inflammatory effect in other cases (141). Local high concentrations of H_2S are also important for inhibiting colon cancer development. Colon cancer cell lines (such as WiDr) and colonic tissues can produce endogenous H_2S through the activity of both CSE and CBS. After 24 h of incubation of WiDr cells with butyrate, production of H_2S was increased and the expression of CBS and CSE upregulated. Both butyrate and NaHS decreased cell viability in a dose-dependent manner. Blockade of CBS, but not CSE, decreased butyrate-stimulated H_2S production and reversed butyrate-inhibited cell viability (93).

2. Endogenous H_2S Production by GI System

Both CSE and CBS have been found in the GI tracts of rats and mice and in the healthy human colon (93, 147, 392, 672). In rat ileum, CBS and CSE mRNA were found (245, 771). While the gastric mucosa expresses both CSE and CBS, CSE appears to play a major role in H_2S generation as PPG inhibits gastric H_2S formation (180). H_2S is released from rat gut tissues (373), such as gastric mucosa (180). H_2S production rate in rat ileum is comparable with that of rat aortas (771). Lysine acetyl salicylate injection into mice (intraperitoneally) increased the endogenous production of H_2S in intestine (586).

H_2S participates in the regulation of various GI functions, from motility control to secretion and inflammation. Similar to its effect on vascular contractility, H_2S inhibits the motility of jejunum and colon in humans, mice, and rats. The precontracted ileum muscles from rats and guinea pigs are relaxed by NaHS in vitro (245, 630). The spontaneous contraction of the isolated ileum tissues from rabbits was also inhibited by NaHS (630). The effects of H_2S on guinea pig gastric antrum muscle strips are more complicated. NaHS at 0.3–1 mM inhibited the spontaneous contraction of gastric antrum muscle strips from guinea pigs. At low concentrations (0.1–0.3 mM), NaHS enhanced the resting tension of muscle strips while slightly reducing the contractile amplitude. The proconstriction effect of NaHS at low concentrations was abolished by 10 mM tetraethylammonium (TEA), a nonselective potassium channel blocker, and 0.5 mM 4-aminopyridine (4-AP), a voltage-gated K^+ (Kv) channel blocker. On the other hand, the relaxant effect of NaHS on the spontaneous contraction of gastric smooth muscle appears to be mediated by the activation of K_{ATP} channels. The opening of K_{ATP} channels and closing of Kv channels by NaHS had also been directly demonstrated on freshly dispersed gastric antrum myocytes using the whole cell patch-clamp recording (770). These observations suggest two different types of ion channels are responsible for the dual actions of H_2S on gastric motility in the guinea pig. Another ion channel involved in the effect of NaHS on mouse colon is SK_{Ca} channels, since apamin at 3 μM inhibited NaHS-induced inhibition of colon motility. It should be noticed that H_2S may also indirectly affect GI smooth muscle contractility by acting on neurons of the enteric nervous system. The presence of H_2S in the mucosa/submucosa of the colon stimulates primary afferent nerve fibers, thus increasing chloride secretion in guinea pig and human (181, 669, 671).

NaHS at millimolar concentrations inhibited rapidly the oxidation of L-glutamine, *n*-butyrate, and acetate in a dose-dependent manner in human colon carcinoma epithelial HT-29 Glc(−/+) cells (351). H_2O_2-caused cell death of rat gastric epithelial RGN1 cells was inhibited by NaHS at 1.5 mM. Once the concentrations of NaHS dropped to 0.5–1 mM, increased cellular toxicity of H_2O_2 was observed. In

anesthetized rats, oral administration of NaHS protected against gastric mucosal lesion caused by ischemia-reperfusion. The antioxidative protection offered by NaHS was mediated by the phosphorylation of ERK or JNK, but not p38 MAPK or K_{ATP} channels (745).

As discussed earlier, H_2S has a double identity as both a proinflammatory and an anti-inflammatory factor. In animal models of gastritis and colitis, H_2S mostly takes an anti-inflammatory role. The protective role of H_2S pathway was investigated in ethanol-induced gastric lesions, which were induced by administration of 50% ethanol for 1 h by gavage (398). Macroscopic and microscopic analyses revealed that NaHS or L-cysteine treatment prevented the gastric damage in a dose-dependent manner. Glibenclamide or capsazepine abolished the protective effects of L-cysteine and/or NaHS. While with K_{ATP} channels and TRPV1 being the molecular targets, sensory afferent neurons might be the cellular targets of H_2S as capsaicin reversed the effects of H_2S (398).

H_2S also regulates gastric mucosal blood flow. With the use of a laser-Doppler flowmetry, it was found that exposure of rat to NaHS (100 μM) increased gastric mucosal blood flow by ~25%, while reducing systemic blood pressure by ~10 mmHg. The activation of K_{ATP} channels in vascular tissue is responsible for this effect of H_2S as it was reversed by glibenclamide (180).

I. H_2S and the Liver

CSE expression is detected in mouse hepatocytes (270) and rat hepatocytes and hepatic stellate cells (180). Both CSE and CBS are expressed in rat hepatic artery, portal vein, and hepatic parenchymal tissues (563). Terminal branches of the hepatic afferent vessels expressed only CSE. After knocking out CSE gene, H_2S production from mouse liver is mostly eliminated (738). This result indicates that CSE is the major enzyme in the liver to produce H_2S. Quantitative comparison analysis also confirmed the dominant role of CSE in the liver by showing a 60-fold lower CBS protein levels in liver than that of CSE. With the provision of the saturated substrates (cysteine and homocysteine), CSE and CBS have the same potency in producing H_2S in liver. However, when the concentrations of cysteine and homocysteine are lowered to physiologically relevant levels and taking the abundance levels of CSE and CBS proteins into account, only ~3% of enzymatic production of H_2S in liver resulted from the activity of CBS (289).

H_2S is an important gasotransmitter in hepatic circulation. Treatment of pentobarbital-anesthetized and laparotomized rats with Na_2S increased the hepatic arterial buffer capacity to 27%, which is ~16% in untreated animals. PPG treatment of the animals, on the other hand, reduced the buffer capacity to 8.5%. Glibenclamide completely reversed the H_2S-induced increase of buffer capacity to the control level, indicating a mediating role of K_{ATP} channels in the hepatic vascular effect of H_2S (563).

The role of H_2S in regulating the intrahepatic circulation and portal hypertension has been investigated in normal and cirrhotic rats (180). Cirrhosis in rats was induced by bile duct ligation. NaHS treatment of the rat suppressed norepinephrine-induced increases in intrahepatic resistance and portal pressure. The vasorelaxant effect of NaHS was blocked by glibencamide but mimicked by administration of L-cysteine, suggesting the dependence on K_{ATP} channels of the effects of endogenous H_2S. The limited study did not attempt to detect CSE and CBS in sinusoidal endothelial cells. On the other hand, CSE mRNA and protein were detected in hepatic stellate cells. Therefore, it was believed that H_2S is generated from the fibroblast-like stellate cells in the liver, which can relax in response to H_2S. The contraction of hepatic stellate cells regulates sinusoidal resistance. CSE activity and expression levels in cirrhotic hepatic stellate cells are suppressed, and H_2S production is low. Consequently, intrahepatic resistance is increased and portal hypertension developed due to unregulated contraction around the sinusoids. Application of L-cysteine also fails to lower intrahepatic resistance due to the diminished CSE expression and activity.

Reverse transsulfuration pathway is significantly altered in cirrhosis with reduced production of H_2S and abnormal metabolisms of methionine, homocysteine, and cysteine. The associated homocysteinemia is partially related to reduced expression/activity of CBS and CSE (393, 521). Inhibiting NO synthase did not alter the vasodilating effect of H_2S. Enhancing NO production by increasing sheer stress in hepatic microcirculation also did not affect H_2S production. It appears that in hepatic circulation, NO and H_2S follow different signaling pathways to affect vascular contractility (180). Nevertheless, to conclude that the hepatic effect of H_2S is endothelium independent is still too premature. In fact, endothelial dysfunction caused by hyperhomocysteinemia in rat livers was reversed by perfusion of the livers with Na_2S (146).

In the mouse liver, H_2S donor (IK1001) protects the liver against 60 min ischemia followed by 5 h of reperfusion injury through an upregulation of intracellular antioxidant and anti-apoptotic signaling pathways (274). H_2S-mediated cytoprotection was associated with an improved balance between GSH and oxidized glutathione (GSSG), attenuated formation of lipid hydroperoxides, and increased expression of thioredoxin-1.

Hepatic ischemia-reperfusion (HIR) is common in major liver surgery. Among the most serious complications of HIR is liver failure due to increased oxidative stress level and proinflammatory cytokines (551). H_2S would protect liver

from HIR damage by the virtue of its anti-inflammatory action. In a rat model of HIR, the production of H_2S and CSE mRNA levels in livers were increased, which was considered as a compensatory self-protective reaction (280). While treatment of the HIR rats with PPG deteriorated liver damage, the administration of NaHS attenuates liver injury, as evidenced by the reduction of serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), attenuation of histopathological alterations, decreased production of pro-inflammatory cytokines, and inhibition of cell apoptosis. The improvement of hepatic circulation by H_2S may also help to decrease the production of oxygen-derived free radicals.

In certain cases, H_2S has been portrayed as detrimental to liver function, which is largely related to its pro-inflammatory action. Endotoxemia is one of those cases with liver damage. LPS-induced acute endotoxemia of Wistar rats caused both circulatory failure (hypotension and tachycardia) and liver damage. Serum levels of ALT and AST were all increased. In the liver, the expression and activity of CSE and CBS as well as myeloperoxidase (MPO) activity were enhanced. Pretreatment of the rat with PPG (10–100 mg/kg iv) abolished endotoxemia-related decrease in liver H_2S production and reversed the increase in MPO activity as well. This effect of PPG is organ specific, since LPS-induced circulatory failure is not affected (122).

J. H_2S and the Urinary System

Predominant CSE expression has been observed using immunohistochemical analysis in mouse kidney cortical tubule (270). CSE abundance has also been shown in rat inner cortex and outer medulla, especially significant in cells of the proximal straight tubule. A quantitative analysis shows a 20-fold lower protein level of CBS than that of CSE in kidney. However, with sufficient substrate provided, CBS would produce much more H_2S than CSE does in the kidney (289).

The effects of H_2S on kidney are mostly manifested with changes in vascular function and tubular functions. Among its tubular functions is the increased glomerular filtration rate (GFR) and decreased tubular sodium reabsorption (42). In anesthetized Sprague-Dawley rats, intrarenal arterial infusion of NaHS increased renal blood flow (RBF), GFR, urinary sodium excretion [U(Na) x V], and potassium excretion [U(K) x V] much more than RBF and GFR, implying that H_2S may directly affect renal tubular functions (723). Infusion of AOA or PPG alone had no effect on renal functions, but together these two agents decreased GFR, U(Na)x V, and U(K)x V, indicating that H_2S produced by CSE and CBS has the similar renal effects on exogenously administered NaHS. These studies show the capacity of H_2S to stimulate natriuresis and diuresis. The tubular effect of H_2S is mostly mediated by the inhibition of $Na^+/K^+/Cl^-$ cotransporter and Na^+-K^+-ATPase activity (723).

A porcine kidney ischemia reperfusion animal model was made by using intra-aortic balloon-occlusion procedure. During reperfusion, norepinephrine was titrated to maintain blood pressure at baseline levels. The application of Na_2S to these anesthetized, ventilated, and instrumented pigs partially restored kidney function such that creatine clearance was improved and high creatinine level in the blood was reduced. The glomerular histological injury as assessed by the incidence of glomerular tubularization has also been improved. The renal protective effect of Na_2S in this case is largely due to the anti-inflammatory and antioxidative action of H_2S that reduced kidney oxidative DNA base damage and inducible NOS (iNOS) expression (566).

H_2S protection not only applies to I/R kidney injury incurred as the consequence of systemic ischemia but also to selective I/R injury only to kidney. For example, selective I/R injury to the left kidney of Sprague-Dawley rats caused lipid peroxidation and cell death in the injured left kidney (725). Whether caused by ischemia or secondary to lipid peroxidation, H_2S production in the I/R-injured kidney was significantly reduced. The key H_2S-generating enzyme involved in I/R kidney is CBS, not CSE (725). I/R renal damage can be reversed by the treatment with NaHS. The role of CBS-generated endogenous H_2S was further demonstrated by the renal protective effect of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (494, 725). This compound is a NO scavenger, but it also stimulates CBS activity. With this compound, renal H_2S level was increased and I/R induced lipid peroxidation and kidney damage were decreased. However, the correlation of NO scavenging and H_2S releasing during renal I/R injury remains to be determined.

Decreased endogenous H_2S level has been linked to hyperhomocysteinemia (HHcy)-associated glomerulosclerosis, although the enzymatic basis for this decrease, assumingly to be the reduced CBS expression abundance, cannot be clearly determined yet (552). The mechanism by which HHcy affects CBS activity or expression is also not clear. Regardless, supplementation of NaHS normalized the GFR in mice with HHcy-associated renal damage. Increased activity of matrix metalloproteinase (MMP)-2 and -9, increased expression of desmin and downregulation of nephrin, and apoptosis in the renal cortical tissues of HHcy mice were also reversed by H_2S treatment. Suppressed superoxide ($O_2^{\cdot -}$) production, increased ratio of GSH/GSSG, reduced macrophage infiltration, and inflammation factors have been claimed to underlie the renal productive effect of H_2S (552).

While the aforementioned studies speak for the importance of CBS in the kidney, the functionality of CSE appears to be critical in protecting the kidney from diabetic nephropathy, one of the chronic complications of diabetes. In streptozotocin (STZ)-induced diabetes in rats, CSE expression and

H_2S production levels in renal cortex were significantly reduced, while the levels of TGF-β1 and collagen IV increased (751). At the cellular level, CSE is expressed in cultured rat renal glomerular mesangial cells. High glucose incubation or PPG treatment of mesangial cells duplicates the renal damage observed with STZ-induced diabetes, i.e., increased oxidative stress and cell proliferation. Exposure to high glucose promoted reactive oxygen species generation and cell proliferation, upregulation of the expression of TGF-β1 and collagen IV, and downregulation of CSE expression. Treatment of cultured mesangial cells with NaHS reversed these effects of high glucose but did not alter these parameters with normal glucose. It appears that hyperglycemia inhibits CSE-catalyzed endogenous H_2S production in the kidney, more specifically in glomerular mesangial cells, leading to diabetic nephropathy.

In addition to the kidney, H_2S also targets other organs in the urinary system. H_2S constricts the detrusor muscle of rat urinary bladder (471). This contractile response to H_2S exhibited marked tachyphylaxis. After pretreatment of the muscles with high concentration of capsaicin, H_2S failed to contract detrusor muscles. Antagonists to tachykinin NK1 and NK2 receptor also abolished the H_2S effect. Together, these observations indicate that H_2S stimulates capsaicin-sensitive primary afferent neurons that subsequently release tachykinins.

K. H_2S and Developmental Biology

The expression profile of CSE during different mammalian developmental stages has been elucidated. At the embryonic stage, mouse liver and kidney exhibit low levels of CSE activity (270). As the animal grows, the expression levels of CSE protein and activity increase, which reach the peak at the age of 3 wk old. Thereafter, CSE expression level in the kidney gradually drops, but that in the liver remains stable. At their adulthood, mice have a higher level of CSE expression in renal cortex than in the medulla, especially in the renal tubules of the inner cortex (270). Variable CSE expression patterns in the kidneys of mouse dams during gestation and lactation have also been observed. The chicken embryo as a model organism has been employed in identifying developmental linkage of CSE expression. During avian embryonic development until embryonic day 13, CSE transcripts were expressed in various developing organs including the notochord, eye, neural tube, limb bud mesenchyme, and sclerotomal compartment of the somites. CSE is highly expressed in renal epithelia throughout kidney development, i.e., in the tubular structures of pronephros, mesonephros, and metanephros. It has also been reported that endogenous H_2S in the mouse brain increased after birth, reached a maximum level at 8 wk, and then decreased (168, 169).

Finally, the relationship between endogenous H_2S metabolism and aging has been indicated in different studies. Aging process involves all organs in mammals' body to different extents. Increased cardiovascular morbidity, cross-linking of cardiac and skeletal muscle proteins, mitochondrial dysfunction (475), impaired NO signaling system (735), loss of skeletal muscle mass (475), and endothelial dysfunction (613) are among the undesirable outcomes of aging. Aging-related changes of H_2S metabolism as well as the role played by H_2S in the aging process are not clear. Plasma H_2S concentrations declined in an age-dependent manner in humans over 50–80 years of age (110). In Fisher 344 $\times$ Brown Norway rats aged 8–38 mo old, the expression of H_2S-generating enzymes in aortic tissues were age-dependently increased, and the contractility of the isolated aortic rings from the aged rats was also increased. On the other hand, no age-dependent change was detected in tissue production of H_2S in either aortic or liver tissues. As such, the role of H_2S in aging process, if any, is difficult to assign (495).

Deficiency in CSE activity might be implicated in glutathione depletion in aged lenses. Sastre et al. (537) reported that 56% of old rats in their study showed age-dependent CSE deficiency in the lenses. The lack of CSE expression and activity could be the consequence of oxidative attack during aging process of aged lenses. Inhibition of CSE activity led to glutathione depletion in lenses and cataractogenesis in vitro.

Overall, CSE gene expression is linked to embryogenesis and aging process in selective organs and species. The alteration of CSE expression and consequently changed production of H_2S would impact on the successful advancement of mammalian developmental stages. The expression pattern and intensity of CSE gene has the potential to serve as a novel marker for the development of selective organs. In this regard, it may be interesting to examine the relationship between the altered CSE expression/activity and various diseases associated with abnormal developmental situations.

Our understanding of the developmental correlation of CBS gene expression is limited. Nevertheless, the involvement of CBS in oocyte development has been recognized. The expression profile of CBS during follicular development in female mice showed the ubiquitous expression of CBS in the ovary with the strongest expression in follicular cells at all stages. In late antral follicles, CBS expression was markedly higher in granulosa cells located close to the antrum and in cumulus cells around the oocyte. However, CBS was absent in the oocytes (371). Differences in the CBS expression profile between oocyte and follicular cells may suggest a role for CBS as a mediator in interactions between oocyte and granulosa cells. Knocking down CBS expression in murine granulosa cells with CBS-siRNA led to gradual increase in homocysteine level. It also increased the rate of germinal vesicle-arrested oocytes. The study shows the important role of CBS in regulation of oocyte maturation (371), but

whether the role of CBS is mediated by altered homocysteine level or endogenously produced H_2S has been unclear.

VI. IS H_2S A TRUE OXYGEN SENSOR?

Oxidative phosphorylation accounts for the majority of the energy source in most mammals and is necessary to maintain the redox potential to carry out oxidation-reduction reactions. Molecular oxygen, O_2, is the terminal electron acceptor for oxidative phosphorylation, essential for ATP generation in mammalian cells. Insufficient oxygen supply to the tissues and cells, i.e., hypoxia, is seen in three scenarios. Anemic hypoxia results from a low level of oxygen partial pressure in inspired air. Hypoxemic hypoxia can be induced by anemic hypoxia and/or deficiency in oxygen-carrying capacity, such as primary hemoglobin diseases. The fingerprint of hypoxemic hypoxia is the decreased oxygen partial pressure and oxygen content in the plasma. The third form is ischemic hypoxia, which is the insufficient or discontinued blood supply to organs, tissues, and cells. The damages brought by ischemia are more than decreased oxygen-dependent ATP production. They also encompass the reduced nutrition supply to the cell and the accumulation of harmful metabolic wastes.

Sensing oxygen level is the intrinsic nature of mammals through their oxygen-sensing tissues and cells to determine the strategies for energy production and consumption, fighting for living and survival. The acute oxygen-sensing process is conducted through oxygen-sensing tissues and cells.

Oxygen-sensing tissues include carotid bodies in vasculature, which detect changes of P_{O_2} in the plasma and direct the compensatory reaction to maintain oxygen homeostasis in mammals. The glomus cells, i.e., chemoceptors, of the carotid bodies are oxygen-sensing cells that release oxygen-sensing signals in response to oxygen level change. The oxygen-sensing signals are then carried through afferent terminals in the form of the sensory discharge, which eventually leads to cardiorespiratory reflexes. Not only the conventional neurotransmitters fulfill this signaling role, but gasotransmitters, such as NO and CO, also function the same (335). Other chemoreceptors are found in the gills (neuroepithelial cells) and airways (neuroepithelial bodies) (456).

Chromaffin cells in the adrenal medulla or other ganglia of the sympathetic nervous system are also oxygen-sensing cells. When oxygen level is changed, these neuroendocrine cells correspondingly alter their secretion of epinephrine or norepinephrine into the bloodstream so that the oxygen supply to the affected tissues would be adjusted. Almost all mammalian cells are affected by oxygen level. The difference between O_2-sensing cells and other types of cells is that O_2-sensing cells directly monitor blood and tissue oxygen tension level, and their reaction affects the oxygenation of other types of cells, being located adjacent or remote (335).

The oxygen-sensing capacity of oxygen-sensing tissues and cells must be realized through oxygen-sensing molecules within. What are/is the oxygen-sensing molecule(s), i.e., oxygen sensor(s)? Where are these oxygen-sensing molecule(s) located in our body? And how do these sensors sense oxygen level change? These are questions that have been challenging and stimulating the scientific world for ages.

A. H_2S as an Oxygen Sensor?

A molecule that is produced endogenously and can instantly interact with oxygen to yield corresponding structural and functional changes that consequently trigger a series of cellular and body reactions is qualified as an oxygen sensor. With this proposed definition, one would naturally think of heme, either in its free form or bound form in heme-proteins, to fulfill such a role. H_2S can also be argued for being one of many oxygen sensors or the ONE. Well, let's see how many lines of evidence support this proposition.

1) The net level of endogenous H_2S is affected by oxygen level via multiple mechanisms, such as the oxygen-dependent consumption (453, 454). *2*) The O_2 sensing in fish chromaffin cells was mediated by H_2S, and these fish cells are homologous to mammalian chromaffin cells (458). *3*) Hypoxia and H_2S elicited similar physiological responses in both nonmammalian and mammalian cells and animals (454, 458, 478). *4*) The glomus cells of the carotid body express both CSE and CBS and produce H_2S. RT-PCR showed mRNA for both CSE and CBS in rat carotid body. Immunohistochemistry staining localized CSE to glomus cells (631). CSE is also expressed in neonatal adrenal medullary chromaffin cells of rats and mice. *5*) Hypoxia stimulates H_2S production from these oxygen-sensing cells. *6*) Hypoxia-evoked H_2S generation and hypoxia-evoked catecholamine secretion are greatly suppressed in CSE knockout mice or by CSE inhibitors in wide-type mice (478). *7*) CSE knockout mice do not respond to hypoxia with normal cardiovascular and respiratory reflexes (478). *8*) Inhibition of endogenous production of H_2S in the carotid body also abolished hypoxia-induced hyperventilation (363).

The roles played by CSE and CBS in oxygen sensing, however, are not always consistent. CBS inhibitors, AOA and HA, suppressed hypoxia-induced chemoreceptor afferent activation, but CSE inhibitors, PPG and BCA, failed to do so (363). There are also countering arguments about the mediating role of H_2S in chemoreflexes. Haouzi et al. (228) found that exposure of rats and mice to 60 ppm H_2S caused different respiratory and metabolic responses to hypoxia. While mice with a smaller body mass exhibited ventilatory response to hypoxia and H_2S, rats with a bigger body mass

only have respiratory response to hypoxia, not to H_2S. The reason why mice react to H_2S with hyperventilation was believed to be the consequence of metabolic depression, not a true arterial chemoreflex (228). One may wonder whether endogenously produced H_2S and inhaled H_2S gas produce different chemoreflexes.

In peripheral tissues, such as the vasculature, the effect of H_2S is affected by oxygen level. H_2S caused vasoconstriction of rat aorta at high O_2 levels but relaxed the tissues at physiological O_2 levels (313). The former may be explained by a product of H_2S oxidation, such as sulfite. If this is true, H_2S may be regarded as an oxygen sensor, since it senses the changes in oxygen level and triggers other cellular responses by altering its own property.

On the other hand, "it is unlikely that H_2S contractions are mediated by either H_2S-NO interaction or an oxidation product of H_2S" in hypoxic vascular constriction (HVC) in invertebrates (455). The rationales for such an argument are that in the specifically studied invertebrate tissues, hagfish vascular tissues, NO production is lacking and that H_2S-induced vasoconstriction is enhanced by low P_{O_2}. In their study, the application of Na_2S or hypoxia to the isolated hagfish and lamprey dorsal aortas (DA) and efferent branchial arteries caused vasoconstriction, but not ventral aortas or afferent branchial arteries. HVC in hagfish DA was enhanced by cysteine and inhibited by AOA, but not by PPG, indicating that CBS-generated H_2S is involved in HVC. Oxygen consumption of hagfish DA was altered as the concentration of Na_2S changed. Inhibition or enhancement of endogenous H_2S production, respectively, decreases or increases tissue oxygen level (455, 456).

In other vascular tissues, such as trout efferent branchial arteries (151), toad and duck pulmonary arteries (151), and bovine pulmonary arteries, it appears that H_2S produces both vasoconstriction and vasodilation in a concentration-dependent manner in some vascular tissues, but not in others (455). Would this be a biphasic vascular effect of H_2S related to tissue oxygen level? No firm answer can be given yet. The oxidation theory would argue that oxygen at the physiological level causes the oxidation of H_2S and lowers free H_2S level, which then aids in pulmonary vasodilation. With hypoxia, oxidation of H_2S is decreased, and more free H_2S is available to induce pulmonary vasoconstriction. The primary prerequisite for this theory is that H_2S per se is a vasoconstrictor, which itself invites debate for numerous studies show otherwise. The secondary prerequisite is that a vasoconstrictor role of H_2S only applies to pulmonary artery, which would then nevertheless aid our understanding of the vasodilator role of H_2S at physiological O_2 level in rat aorta (313, 771, 773).

Different vascular tissues across the phylogeny react to hypoxia differently (532, 576). Interestingly enough, these responses are almost exactly mimicked by those elicited by H_2S (151, 152). The inhibition of H_2S production in these vascular tissues inhibits vascular responses to hypoxia, and the promotion of H_2S by supplying additional cysteine greatly promotes the hypoxic response (454). For example, the systemic vascular resistance (R_{sys}) of freshwater turtles significantly increased during anoxia, and so did the plasma H_2S concentration. The contribution of H_2S to this phenomenon in anoxic red-eared slider turtles was studied (593). NaHS infusion increased R_{sys} in living turtles under normoxic conditions. This whole body action was supported by NaHS-induced constriction of the isolated turtle mesenteric and pulmonary arteries with regular oxygenation. Once the animals were exposed to anoxia, NaHS no longer caused vasoconstriction. The application of PPG or hydroxylamine also partially reversed anoxia-induced increase in R_{sys} (593). These observations support the idea that the vascular response to hypoxia is mediated by H_2S.

Beyond the vascular responses, H_2S also mediates hypoxic relaxation of the trout urinary bladder. The oxygen sensor role of H_2S has also been suggested in the kidney. Elevated H_2S level was seen in the renal medulla with hypoxia. This would help increasing medullary blood flow due to the vasorelaxant property of this gasotransmitter. Increased local H_2S concentration would also reduce the energy demand for tubular transport. Consequently, regional O_2 balance would be restored (42).

One mechanism explaining how H_2S senses oxygen levels in the carotid body involves K_{Ca} channels. NaHS induced chemoreceptor afferent nerve discharge in an isolated mouse carotid body/sinus nerve preparation. This effect was antagonized by CO (363). Furthermore, HO-2 participates in hypoxia-evoked H_2S generation in the carotid body (478). As is well known, CO stimulates K_{Ca} channels in different cell preparations (688, 716). Thus a possibility was raised that H_2S may inhibit K_{Ca} channels. To substantiate this view, it was found that the removal of extracellular calcium or the application of K_{Ca} channel blockers abolished NaHS-evoked chemoreceptor excitation. In isolated mouse type I glomus cells, either hypoxia or NaHS inhibited BK_{Ca} currents (363). The similar inhibitory effects of NaHS on native BK_{Ca} channels in rat glomus cells or on human recombinant BK_{Ca} channels has been confirmed using the inside-out single-channel recording technique (631).

The scheme so far elucidated appears to be that hypoxia stimulates CSE or CBS in glomus cells, leading to increased production of H_2S. The latter inhibits BK_{Ca} channels to depolarize cell membrane. Action potential is thus triggered, and the afferent nerve discharges. But the question comes back to the definition of an oxygen sensor, proposed at the beginning of this section. It is convincing that H_2S can cause reactions to hypoxia in oxygen-sensing tissues or cells. It is not convincing that H_2S itself qualifies as an

oxygen sensor because it is CSE or CBS that senses the change in oxygen level and regulates the production of H_2S. The activities of CBS and CSE may be affected by the oxygen level (Po_2) or oxidative status of the cells (383, 598). In this way, altered oxygen level can impact on cellular H_2S production. CBS itself may not bind oxygen, but it can be quickly oxidized from the ferrous to the ferric state via an outer sphere electron transfer (28). This rapid and reversible oxidation would depend on the redox potential of the heme in CBS, which has been unknown. Nevertheless, a low redox potential of CBS is expected due to the presence of the cysteinate ligand (28). The interaction of CSE with oxygen is not clear. Between CSE and CBS, it is not sure which enzyme should take the credit for oxygen sensing. CBS is a heme protein, and as such would be rationalized for its reaction with oxygen. Then, how does CSE sense the change of oxygen level? Is H_2S or CSE/CBS the real oxygen sensor? The answer may come from a new line of evidence whether a change in oxygenation level actually alters the structure or function of H_2S molecule per se.

B. Oxygen-Dependent H_2S Consumption/Oxidation

The oxidation of H_2S to sulfate, by using O_2, has been known as far back as 1921 when the oxidation of H_2S in blood was described by Haggard in 1921 (226). Smythe (577) also noted in 1942 that two products of cysteine desulfuration by liver, ammonia and pyruvate, were formed in equal amounts aerobically or anaerobically while the recovered H_2S was in much smaller amounts in aerobic experiments. Sulfide oxidation is an important adaptive reaction and protective mechanism for intertidal invertebrates, such as the sipunculid worm *Phascolosoma arcuatum,* the mudskipper *Boleophthalmus boddaerti,* and the nematode *Oncholaimus campylocercoides* (266). With low oxygen level and high sulfide level in their living environment, these invertebrates store or excrete products in the oxidation state of elemental sulfur during hypoxia. Sulfite and sulfane sulfur have already been identified as intermediates of mitochondrial sulfide oxidation for the mussel *Solemya reidi* (445). H_2S oxidation also becomes especially relevant in the detoxification process in colonic mucosa, where large quantities of H_2S were generated by colonic bacteria.

Much of our knowledge about H_2S oxidation was initially derived from sulfide-adapted bacteria or invertebrates including polychaetes, crustaceans, and bivalves and the lugworm *Arenicola marina*. In mammals, H_2S is oxidized initially to perfulfide and subsequently to thiosulfate and sulfite and finally to sulfate (39, 130, 238). While the liver and colon tissues appear to be mostly active in H_2S oxidation, almost all cells have the capacity to do the same. In short, three consecutive steps constitute the mitochondrial sulfide oxidation pathway with three key enzymes involved. It starts with the production of persulfide by sulfide: quinone oxidoreductase (SQR) followed by the formation of sulfite due to sulfur dioxygenase. The final step of sulfide oxidation is catalyzed by sulfur transferases. These key enzymes needed for H_2S oxidation are all putatively located in the mitochondria (195).

SQR, a mitochondrial membrane flavoprotein, is the first critical enzyme in mitochondria to catalyze the oxidation of H_2S. SQR gene has been detected in human and mouse genomes, and its functional relevance was revealed in both invertebrate and mammalian mitochondria (635). Its function has also been characterized by heterologous expression of *A. marina* SQR in yeast (636). In both rat liver mitochondria and *A. marina,* H_2S oxidation is completely blocked by myxothiazole, emphasizing the irreplaceable role of SQR in the process (662). However, SQR activity in rat mitochondria is about four times lower than that in *A. marina,* reflecting the high intolerance of the lugworm to H_2S.

SQR in mitochondrial membranes transforms H_2S to protein-bound persulfide. Hypothetically, SQR functions as an acceptor with a persulfide bound to its cysteine residue (636). This step simultaneously reduces equimolar concentrations of decyl ubiquinone. Ubiquinone receives two electrons for the oxidation of each sulfide molecule in this step and transfers them to complex III (636).

The conversion of SQR-bound persulfide to sulfite is proposed to be catalyzed by a putative sulfur dioxygenase. Sulfur dioxygenase appears located in the mitochondrial matrix. This enzyme would catalyze the subsequent four-electron oxidation of one persulfide molecule to sulfite. Again, SQR may transfer the sulfane atom produced during the conversion of persulfide to sulfite to a cysteine residue of the sulfur transferase. Subsequently, sulfur transferase (or rhodanese) adds the second persulfide to sulfite to complete this process with the production of thiosulfate in the mitochondrial matrix (650). This scheme is a replica of the double displacement mechanism originally proposed for the rhodanese reaction (698). The importance of sulfur transferase in sulfide oxidation has been strongly indicated. The K_m values for sulfite and persulfides of the rhodanese activity of the purified sulfur transferases from rat and lugworm (35, 238, 689) are within physiological range. Deficiency in rhodanese activity in colonocytes of patients with ulcerative colitis and colorectal cancer is linked to the elevated intestinal sulfide concentration, suggesting the accumulation of toxic sulfite (506). It should be aware that, however, sulfur transferase in mammals is also engaged in other metabolic functions in addition to sulfide oxidation (238).

Different from the invertebrates (e.g., *Arenicola marina)* in which thiosulfate is the final product of sulfide oxidation and is excreted from the body (661), rat liver mitochondria can further convert thiosulfate to sulfate via the actions of

thiosulfate reductase (417) and sulfite oxidase (121, 238). Sulfite oxidase, a heme protein, can also directly oxidize sulfite to sulfate. Sulfate is the major end-product of H_2S metabolism under physiological conditions. It constitutes 77–92% of total urinary sulfur (39).

Additionally, low-molecular-weight thiols such as GSH or dihydrolipoate or thioredoxin may transfer the SQR-bound persulfide to sulfur dioxygenase (431, 659). This is likely to happen since glutathione persulfide is a substrate for the dioxygenase (642). Sulfur dioxygenase has been shown to oxidize glutathione persulfides to thiosulfate in the sulfur bacteria *Acidothiobacillus thiooxidans*, *Acidothiobacillus ferrooxidans,* and *Acidiphilium acidiphilum* (523).

As a guarded note, many of these enzymes involved in H_2S oxidation have not been completely purified to identify their sequences or fully characterized in mammalian mitochondria. As such, the efficiency and effectiveness of H_2S oxidation as well as the proposed oxidation mechanism in mammals should be considered with a great cautiousness (636).

The capability of mitochondria to oxidize H_2S leads to a conventional assumption that H_2S is produced in the cytoplasm and consumed in mitochondria (89, 546). This convention is not entirely correct. MST/CAT-mediated H_2S production may occur inside mitochondria should 3-MP exist. Translocation of CSE and CBS from cytosol to mitochondria is also a legitimate possibility. Nevertheless, Olson et al. (454) proposed that "the actual concentration of biologically active H_2S is determined by the simple balance between H_2S production and the amount of O_2 available for H_2S oxidation, such as tissue Po_2." The lower Po_2, the slower H_2S oxidation. That leaves more free H_2S to perform its physiological function. Oxygen partial pressure in rat renal cortex is close to that in the renal vein (70 mmHg), but becomes very low in the renal medulla, between 5 and 15 mmHg (164). In this hypoxic environment, hypoxia-induced factor-1 (HIF-1), iNOS, COX-2, and HO-1 are expressed at higher level than in the cortex, and H_2S levels are also believed to be higher (42). This regional high level of H_2S would help increase medullary blood flow and restore O_2 balance.

In an experiment on minced trout heart at near zero Po_2, H_2S production was decreased transiently by injection of micromolar amounts of O_2. The transient decrease in H_2S level could be due to the oxidation of existing H_2S or inhibition of H_2S-generation enzyme. Because this only occurs at zero basal Po_2 (454), whether this phenomenon applied to the heart under physiological conditions is not sure. Fish heart and mammalian heart also may not share the same oxygen-sensing mechanism. The notion that eukaryotes utilize oxygen to oxidize H_2S also meets with criticism. For example, inhalation of low level H_2S rendered the mice consumed 10-fold less oxygen and reduced their basal metabolic rate (56). In this case, the cells do not use more oxygen to oxidize the newly increased H_2S.

C. H_2S-Related Oxygen Consumption and ATP Production

The contribution of electrons through ubiquinone to the respiratory chain during H_2S oxidation would favor chemolithotrophic ATP production in sulfide-adapted invertebrates, such as *A. marina* and the mussel *Geukensia demissa* (149, 215, 662). Other examples of animal species who possess the ability to utilize sulfide directly as an inorganic energy source during mitochondrial sulfide oxidation include *S. reidi* (493), *Fundulus parvipinnis, Citharichthys stigmaeus* (22), and *Heteromastus filiformis* (447). Sulfide oxidation and oxidative phosphorylation are coupled at the cytochrome *c* oxidase to stimulate ATP production. The ratio of mole ATP production per mole mitochondrial sulfide consummation in these animal species was measured from 0.5 to 1.25. This ratio is clearly lower than the estimated potential value of 2.0–4.3 mol ATP per mol sulfide when sulfide oxidation occurs outside the mitochondrial inner membrane. This difference in part may be due to an uncoupling effect of the H_2S molecule. "The low efficiency of ATP coupling during sulfide oxidation may be profitable considering the necessity of sulfide detoxification. Loosely coupled mitochondria will allow a more rapid sulfide oxidation when independent from cellular ATP utilization" (445).

H_2S can also be used as an inorganic substrate for energy production in human cells. For example, in rat liver mitochondria, sulfide oxidation and oxidative phosphorylation are coupled with the respiratory control ratios >1 (217). The physiological implication of the oxidation of H_2S to energy metabolism in mammals is rationalized as rat and lugworm SQR have the low K_m values for sulfide (2–26 μM) (217). Within this physiological range, H_2S can stimulate oxygen consumption and increase membrane potential. In isolated chicken liver mitochondria, application of H_2S from 10 to 60 μM increased the consumption of O_2. ATP production in mitochondria in this preparation was also linked to H_2S oxidation. Maintaining H_2S concentration in the isolated mitochondria preparation at <5 μM becomes a prerequisite for ATP synthesis (748).

Whether ATP provision from sulfide oxidation could be a contributing factor to energy supply in living animals, even in intact cells or tissues for that matter, remains unsettled. Experimental data have shown that oxygen consumption rates of some symbiont-free animals are enhanced during exposure to low sulfide concentrations (157). The study on the mussel *Geukensia demissa* who lives in high sulfide sediments may be relevant in this regard (342). Serotonin increased oxygen consumption rate of excised gills from *G.*

demissa in addition to its stimulatory effect on ciliary beating. Sulfide also stimulates the oxygen consumption rate at concentrations up to 1 mM (342). Blockade of complex III in the gills' respiratory chain with antimycin A suppressed both oxygen consumption and ciliary beat frequency. Although sulfide alone would have inhibited ciliary beat frequency (204), supplementation of sulfide reversed the inhibitory effects of antimycin A. It appears that sulfide acts as an alternate substrate for oxidative phosphorylation to compensate the inability of electrons from oxidation of endogenous substrates to reach cytochrome *c* oxidase (204). In another marine polychaete worm *Marenzellaria viridis*, the metabolic heat production increased in the presence of sulfide (544). The aforementioned studies may be used as evidence for the capacity of sulfide oxidation using oxygen in vivo or for the potential of sulfide as an alternative substrate for energy production, but they do not demonstrate that energy supply is actually enforced by H_2S. Neither such an energy supplementation role is substantiated for endogenous H_2S.

It should be kept in mind that sulfide-based ATP production has low efficiency than using other substrates as the terminal electron acceptor, like succinate or malate (22, 493, 662). Therefore, with low cellular oxygen level, mitochondrial sulfide oxidation would further exhaust oxygen supply, which is not a good thing for the cells. It is equally important to note that in certain types of cells, more to the point of concentration dependence, H_2S might inhibit oxygen consumption at cytochrome oxidase level (215, 748). Higher concentrations of H_2S also inhibited the oxidative phosphorylation in the isolated mitochondria from chicken liver (748). In human colon carcinoma epithelial HT-29 Glc(−/+) cells, NaHS caused 50% inhibition of cellular respiration at ~30 μM and reduced O_2 consumption. This would partially compensate for the reduction in ATP synthesis and maintain a constant energetic load, thus preserving cell viability (351).

D. H_2S and Chronic Oxygen Sensing

The chronic oxygen-sensing process involves the activation of the transcription factor HIF-1. The latter event triggers the oxygen-regulated gene expression and protein synthesis so that adaptive physiological responses can be mobilized to cope with the new oxygen level. This is an intrinsic mechanism for all oxygen-consuming cells. HIF-1 is rapidly degraded under normoxic conditions from an oxygen-dependent hydroxylation event and subsequent ubiquitin-dependent degradation (163). Hypoxia stabilizes HIF-1 due to the inhibition of HIF prolyl-hydroxylase. As the HIF-1-regulated functional change requires the synthesis of new proteins, the cellular reaction time to hypoxia through this pathway is rather slow. Both H_2S and hypoxia increased HIF-1 protein concentration and nuclear localization. The latter is positively correlated with the survival of *C. elegans* exposed to H_2S (77). The presence of HIF-1 is absolutely needed for animals' response to H_2S, since no worms survived after exposure to 15 ppm H_2S in the ambient air if HIF-1 is genetically knocked out. However, the involvement of HIF-1 in the chronic hypoxia sensing of H_2S is complex. For one thing, H_2S and hypoxia caused different expression patterns of a HIF-1 reporter gene. For another, the degradation of HIF-1 in *C. elegans* is mediated by the von Hippel-Lindau tumor suppressor (VHL)-1 ubiquitin ligase. Hypoxia-induced stabilization of HIF-1 is mediated by VHL-1, but that of H_2S is independent of VHL-1 (77). Therefore, it seems that H_2S may not be involved in the protein stabilization of HIF-1 during chronic oxygen sensing in *C. elegans*.

On the other hand, egl-9 mutant worms have a greater HIF-1 reporter activity and tolerate higher H_2S concentrations than VHL-1 mutant worms (77). While VHL-1 is not required for the H_2S-induced expression of a reporter for HIF-1 activity, egl-9 is needed. An involvement of H_2S in the transcriptional activation of HIF-1 is thus suggested. Previous studies have already provided evidence that, in addition to HIF stability, HIF activity can be regulated. Growth family member 4 (ING4)-dependent HIF regulation is one of these examples (464). HIF prolyl hydroxylases, EGLN1, is another example which represses HIF-1α transcriptional activity in hypoxia (644).

The role of H_2S in chronic oxygen sensing has also been investigated in mammalian cells. Cultured rat brain capillary endothelial cells were treated with cobalt to mimic hypoxia condition, and the effect of NaHS on cell function was tested (376). It was found that NaHS stimulated endothelial cell proliferation and migration under hypoxic stress. These pro-angiogenic effects were related to NaHS-induced upregulation of HIF-1α mRNA and proteins. HIF-1α binding activity under hypoxic condition was also increased by NaHS. It appears from this study that the degradation of HIF-1alpha was not affected, but its synthesis was promoted, by H_2S. Again, this would not fit into the action frame of hypoxia. Furthermore, cobalt treatment could mimic hypoxic stress, but the model itself is not hypoxia in a strict sense as cobalt treatment also induces many other cellular changes such as the upregulation of HO-1. In short, an oxygen-sensing role of H_2S in chronic hypoxia cannot be established in mammals yet.

VII. H_2S, HIBERNATION, AND AGING

A. Inducible Suspended Animation: From Small to Large Animals to Humans

A suspended animation state, hibernation, occurs naturally in many mammals including bats, chipmunks, echidnas, possums, hedgehogs, hamsters, skunks, prairie dogs, mar-

mots, badgers, some lemur, and some rodents (e.g., mole rats). Among nonmammals, species of lizards, frogs, toads, newts, snakes, turtles, and insects also hibernate. Hibernating animals get their energy by gluconeogenesis. Warm-blooded creatures, like mammals and birds, normally keep their core body temperature constant, independent of the changes in environmental temperature, while exceptions including suspended animation-like states such as hibernation, torpor, and estivation occur mostly in cold-blooded animals. These states are featured by significant reductions in metabolic rate, followed by a loss of homeothermic control.

Hibernation refers to an adaptive reduction of energy utilization through reduced activity when the energy supply is reduced. Anoxia induced a hibernation of zebrafish *Danio rerio* embryos (466). The application of CO induced a suspended animation of *C. elegans*, which protected the species from hypoxic damage (443). In 2005, researchers in Seattle made a news headline that they induced a suspended animation-like state in the house mice (*Mus musculus*) by allowing the animals to inhale 80 ppm H_2S (56). This is an important discovery since mice usually do not hibernate, but can fall into a state called clinical torpor in the condition of food shortage. H_2S exposure dose-dependently reduced metabolic rate by 50% with a 90% decrease in oxygen consumption. The breathing rate of the animals sank from 120 to 10 breaths/min, and their body temperature fell from 37 to as low as 15°C, as the animals had, in effect, been made cold-blooded. Animals ceased all movement and survived like this for 6 h. After cessation of H_2S exposure, the mice awoke without displaying any neurological or behavioral deficits. Further experiments showed that pretreatment with H_2S increased the survival rate of mice exposed to hypoxia, whereas hypoxia alone led to death of all control mice (57). A pretreatment of the mice with 150 ppm H_2S for only 20 min dramatically prolonged the period of survival. These pretreated mice could endure 5% oxygen and survived for more than 6 h, whereas untreated mice survived for $<$15 min at this oxygen level. Pretreatment of the mice for 20 min with 150 ppm H_2S, followed by 1 h at 5% oxygen and another 1 h at 3% oxygen, enabled the mice to survive for several hours at 3% oxygen. This could be explained by H_2S-induced reduction in oxygen demand under hypoxic conditions.

The H_2S-induced suspended animation in nonhibernating mice was also achieved by others using the same experimental set up with awakened mice, exposed to 80 ppm of H_2S in a warmed environment (663). The cardiovascular and metabolic effects of inhaled H_2S were further examined using telemetry and echocardiography in conscious mice. Echocardiography demonstrated that H_2S inhalation at 27^0C for 6 h significantly decreased heart rate and cardiac output, but stroke volume was not affected. A near 50% heart rate drop was observed during H_2S inhalation, and it was reversed within 30 min termination of H_2S inhalation. The induced bradycardia may result from the inhibitory effect of H_2S on sinus node activity. Blood pressure and blood oxygen level remained unchanged. Meanwhile, core body temperature, respiratory rate, and physical activity were all reduced. These effects of inhaled H_2S seem not to be related to body temperature change, since inhaling H_2S for 6 h at 35°C ambient temperature produced the same body reaction as H_2S did at 27°C, except the constant core body temperature. As the hypothermia under this condition had been prevented, the H_2S-induced suspended animation was unlikely regulated by body temperature (663). In fact, the hypometabolism was achieved by H_2S inhalation before the body temperature drop. The relationship between body temperature and the effect of inhaled H_2S was also reported in another experimental set where H_2S inhalation (80 ppm) was proven to be protective against mechanical ventilation-induced lung injury in mice. This inhalation indeed decreased body temperature from 36.0 to 34.0°C during mechanical ventilation. However, merely producing mild hypothermia (34°C) did not reduce ventilator-induced lung injury, and the H_2S protection was not altered by changing animals' body temperature from 36 to 34°C. It was concluded that H_2S inhalation in low doses prevents VILI in mice that is independent of the reduction of body temperature (170). A similar conclusion was reached in rats where NaHS infusion protected the lungs from VILI (14). Inhalation of H_2S (100 ppm) during anesthesia and mechanical ventilation exerted anti-inflammatory effects on the mouse lungs against sepsis damage. But this protection was not enhanced by changing core temperature of the mice (38°C) to deliberate hypothermia (27°C) (668).

Rather than a body temperature-related mechanism, decreased whole body oxygen consumption and carbon dioxide production, the indication of reduced metabolic rate, are more likely responsible for the hibernation act of H_2S. A hypometabolic status would optimize the balance between oxygen supply and demand through the effect of H_2S on key molecules involved in energy metabolism. As known, H_2S is a reversible inhibitor of cytochrome *c* oxidase with similar effects as other inhibitors (39). The inhibition of cytochrome *c* oxidase may curb the oxygen demand for the animal and make it less dependent on supply. In stressful situations, such as low oxygen supply, if the reduction of supply is preceded by suppressed demands such as pretreatment with H_2S, then reactive oxygen species should not be generated as much and cell damage should be attenuated.

Exogenous H_2S was given through injection to induce suspended animation in rats. In anesthetized and mechanically ventilated rats, infusion of NaHS solution (2 $mg \cdot kg^{-1} \cdot h^{-1}$) induced hypometabolism with decreased heart rate and exhaled CO_2, lower body temperature, and lower respiratory rate (14, 15).

The above studies were all conducted in rodent models. Rodents have large surface area-to-mass ratios so that their core temperature can be altered relatively fast. In larger animals and humans, such an adjustment in core temperature is more indisposed. It becomes essential to examine, therefore, whether H_2S is also capable of inducing suspended animation in larger animals and humans. Such an effort has been undertaken, and the results are rather perplexing.

In one study, the anesthetized piglets inhaled H_2S via mechanical ventilation with a constant ambient temperature of 22°C. The dosage of H_2S ranged from 20 to 80 ppm for 6 h. Indeed, core temperature and oxygen consumption were significantly decreased, but mean arterial pressure increased. Heart rate, cardiac output, and lactate were not changed at all. The researchers concluded that "H_2S does not appear to have hypometabolic effects in ambiently cooled large mammals and conversely appears to act as a hemodynamic and metabolic stimulant" (358).

This observation on piglets was not in agreement with the experiments on pigs. In anesthetized pigs, Na_2S solution was first given as a bolus of 0.2 mg/kg iv, followed by an infusion of 2 $mg \cdot kg^{-1} \cdot h^{-1}$. This treatment decreased O_2 uptake and CO_2 production, slowed heart rate and decreased cardiac output, and lowered body temperature (567). A hypometabolic effect appears to be achieved. Would it make a difference whether H_2S is given via inhalation (358) or infusion (567)? One thing for sure is that the dosages of H_2S the animal received in these studies are difficult to compare without actual measurement of H_2S level in animals' circulation.

Let's scale up the study further to sheep. Haouzi et al. (229) successfully replicated H_2S-induced suspended animation in mice, but they failed to play the same trick with sheep. The animals breathed spontaneously and lied calmly on their side under the sedation with ketamine. H_2S was inhaled at 60 ppm, and metabolic rate was measured. However, these sheep did not react to H_2S with the induction of hypometabolism.

Several reasons may underlie the differences between smaller and bigger nonhibernating animals in their hypometabolic responses to H_2S. First, difference in surface area-to-mass ratios may render larger animals more resistant to core temperature change. However, we already know that in larger animals H_2S is able to lower the core temperature but failed to decrease metabolism. At any rate, there seems no correlation between core temperature and hypometabolism induced by H_2S. Second, differences in body mass may impact on the metabolic responses to H_2S. Haouzi et al. (228) exposed small and large rodents (20 g mice and 700 g rats) to 60 ppm H_2S and examined the animals' respiratory and metabolic changes. H_2S and hypoxia profoundly decreased metabolic rate in the mice, but not in the large rats. The onset of the hypometabolic effect of H_2S on mice was faster than that of hypoxia. Hypoxia, on the other hand, stimulated ventilation but not metabolism, while H_2S affected neither breathing nor metabolism of rats (228). Third, higher dosages of H_2S may be required to induce hypometabolism in larger animals and humans. This approach could be problematic or even dangerous considering the toxicological profile of H_2S at high concentrations. Olfactory neuron loss and nasal lesions have been reported after rats inhaled H_2S at dosages ranging from 30 to 80 ppm (66). Human exposure to ~20 ppm H_2S was reported with symptoms of diffused neurological and mental behavior (297). Fourth, anesthesia may alter the body response to H_2S. In experiments with larger animals (piglets or sheep) anesthesia was used, while smaller animals (mice mostly) were conscious with spontaneous respiration.

Whereas the exploration continues, H_2S-induced hibernation in humans, if possible, would offer extremely promising opportunities for emergency management of severely injured patients, for saving life when facing natural disaster or war, for obtaining invaluable time to find solution for those end-stage patients, and for the conservation of donated organs. The list of applications goes on and on.

Sustained deep hypothermia (31°C) was induced in 17-mo-old Sprague-Dawley rats after exposing the animals to inhaled H_2S for 48 h. This hypothermia protected the animals from the preceding stroke with a 50% reduction in infarct size in the brain (183). The expression of pro-inflammation or pro-apoptosis genes (caspase-12, NF-κB, and grp78) in the peri-infarcted region was downregulated and the animals showed better performance on memory and learning tests. These beneficiary outcomes may result from H_2S-induced hypothermia as the researchers believed, but they may also be directly caused by H_2S-induced hypometabolism independent of body temperature change. At the patient level, mild hypothermia (32–34 °C) improves neurological outcome of the patients with a cardiac arrest. Ischemic brain injury is manifested with cell swelling, apoptosis, and global cerebral dysfunction (216).

Clinical studies also showed that several clinic subgroups of patients developed a cardiac metabolic adaptive reaction, namely, myocardial hibernation (505, 543). In these patients, physiological ischemia "hibernates" the cardiac tissues with reduced myocardial contractile function and thus reduced energy deficit. Once the blood supply to the heart is restored through reperfusion/revascularization, the cardiac contractile function recovered. It is not clear why only a fraction of patients have this cardiac reaction, but certainly a hypometabolic status in the heart would be welcomed for the ischemic heart. H_2S may be the agent for this purpose.

At the individual organ level, the success of organ transplantation might be aided by H_2S. Among many factors that affect the viability of the donor organ is the donor management prior to organ procurement and the duration of hypothermic storage (432). An ideal preservative solution for the donor organ and low temperature storage during transportation are acknowledged strategies of choice. The H_2S-induced "hibernation on demand" would be used as a principle to reserve donor organs based on the consequent hypometabolism of the organ. An attempt in adding 1 μM NaHS to the organ preservation solution has been made for isolated rat hearts (249). The addition of NaHS improved ATP production and reduced cell apoptosis better than St. Thomas solution did. This will open the door for prolonging the viability of the donor organs for waiting and transportation time.

B. Blood Transfusion: Reduced Need by H_2S

H_2S has been used to reversibly reduce metabolic demand during excessive blood loss, thus in a state of low oxygen supply (413). In an extreme case, Sprague-Dawley rats lost 60% total blood as a controlled but unresuscitated hemorrhage model. Only 14–23% of the bled animals survived longer than 24 h after hemorrhage. Over the same period and with all other experimental conditions being the same, inhalation of H_2S gas or intravenous infusion of NaHS increased the survival rate to 67–75%. The H_2S-survived animals behaved normal and their respirometry analysis showed stable metabolism during and after hemorrhage. Ganster et al. (201) examined the effect of NaHS treatment (0.2 mg/kg, a bolus injection intravenously) on resuscitated hemorrhagic shock in rats. Bleeding for 60 min lowered the animals' mean arterial blood pressure (MAP) to ~40 mmHg. NaHS treatment of these rats 10 min before retransfusion of shed blood greatly helped maintain MAP and carotid blood flow. Hemorrhagic shock-induced metabolic acidosis, iNOS expression, and NO production in the heart and aorta were significantly inhibited by NaHS. The expressions of Nrf2, HO-1, and HO-2 were also increased by NaHS in both aorta and heart. The clinical application of this effect of H_2S against hemorrhage, observed on rodents, is of great interest and importance.

In contrast, Mok et al. (408) found that the unresuscitated rats that underwent hemorrhage shock rapidly restored their MAP and heart rate and reduced organ injury after being treated with PPG, suggesting that endogenous H_2S might be detrimental for hemorrhage shock. The reason for this conflicted observation with other studies reporting the life-saving effect of exogenous H_2S supplementation for hemorrhage is not readily explainable.

C. Prolonged Lifespan: Studies with *Caenorhabditis elegans*

Caenorhabditis elegans has been used widely in studying the secrets behind the prolonged life span. Miller and Roth (402) grew *C. elegans* in an atmosphere containing 50 ppm H_2S. These animals exhibited normal phenotype without signs of metabolic inhibition and without abnormal embryonic or postembryonic behavior. However, they became thermo-tolerant. At high temperature, animals grown in H_2S showed an average of 77% survival rate while during the same time period all untreated animals had died. *C. elegans* exposed to H_2S also had a longer lifespan. Compared with the untreated animals, the H_2S-treated animals lived by average 9.6 days longer, a 70% increase. Another interesting phenomenon is that a slower aging process of the animals requires the life-long exposure to H_2S. The survival mechanisms in this case are not related to the insulin signaling pathway, mitochondrial dysfunction, or caloric restrictions. Nevertheless, the mediating link has been pointed to the increased SIR-2.1 activity by H_2S. SIR-2.1 is capable of translating environmental change into physiological alterations that improve survival of many organisms, including *C. elegans* (61). In contrast to wild type, overexpression of SIR-2.1 increases lifespan of *C. elegans* by 18–50%, which depends on the activity of forkhead transcription factor (daf-16) (643). H_2S treatment also increased the lifespan of *C. elegans,* but independent of daf-16 (402). Furthermore, H_2S treatment of the animals did not increase SIR-2.1 transcript levels. Therefore, it is not the expression of SIR-2.1 that can explain the effect of H_2S. Deletion of SIR-2.1, in contrast, abolished the H_2S-induced thermotolerance and lifespan extension of nematodes. Taken together, these observations suggest an increased SIR-21 activity is responsible for H_2S-induced lifespan extension thermo-tolerance.

The mechanisms for the effect of H_2S on aging process appear quite different from that for H_2S-induced suspended animation. As such, the applications of these results to human physiology and pathophysiology would also be different. By exposing the animals to the same range of H_2S (50–80 ppm), *C. elegans* have an unchanged general metabolism (402), but mice react with hypothermia and hypometabolism (57). We simply cannot leave the species difference unattended in this case as it appears to really matter. Can we slow the aging process by using H_2S (boosting endogenous one or supplying exogenous one) in mice, rats, pigs, sheep, and all way to humans? This is challenge we all want to explore.

VIII. PATHOPHYSIOLOGICAL IMPLICATIONS OF ABNORMAL H_2S METABOLISM

A. Angiogenesis

In its loose definition, angiogenesis refers to the spontaneous blood vessel formation and/or the growth of new blood

vessels from preexisting vessels. This process is vital not only for physiological events in normal growth and development but also for pathophysiological situations such as tumor development, wound healing, or ischemic cardiac infarct as well. Angiogenesis involves several sequential phases during which endothelial cell migration might play a major role. The role of H_2S in angiogenesis can be exemplified by the impact of H_2S on endothelial cells. NaHS (10–20 μM) treatment of cultured RF/6A endothelial cells endothelial cells led to cell proliferation, adhesion, migration, and tubelike structure formation on Matrigel. The underlying mechanism was believed to be the activation of PI3K/Akt, since the pro-angiogenesis effect of H_2S was abolished in the presence of PI3K inhibitor LY294002 and wortmanin (88). The same effects of H_2S were observed with cultured bovine umbilical vein endothelial cells (467). The increased phosphorylation of Akt, ERK, and p38 was observed in the presence of H_2S. However, since glibenclamide blocked H_2S-triggerd p38 phosphorylation and endothelial cell motility, it was proposed that K_{ATP} channel activation plays a critical role in the whole process. Moreover, the pro-angiogenesis effect of vascular endothelial growth factor (VEGF) is mediated by H_2S, since not only VEGF released H_2S from vascular endothelial cells but its effect on migration of endothelial cells was also attenuated by silencing CSE expression. Although the application of CSE inhibitors, such as PPG or BCA, in this study also inhibited the vascular growth of chicken chorioallantoic membranes, the pharmacological manipulation of CSE activity cannot be used as concrete evidence for the involvement of endogenous H_2S. The researchers then used CSE knockout mice to isolate aortic rings. These rings under in vitro incubation conditions exhibited markedly reduced microvessel formation in response to VEGF compared with wild-type littermates. Topical administration of H_2S also enhanced skin wound healing in living rats, which was also significantly delayed in CSE KO mice. These probably are the most convincing evidence and the most important contribution to establish the pro-angiogenesis role of endogenous H_2S (467).

The benefit of establishing new collateral blood vessels to the ischemic tissues or organs is obvious. In this regard, the hindlimb ischemia model has been used by femoral artery ligation. In a rat model, NaHS treatment for 4 wk significantly increased collateral vessel growth, capillary density, and regional tissue blood flow in ischemic hindlimb muscles compared with controls. Increased VEGF expression in ischemic hindlimb muscles and the phosphorylation of VEGF receptors as well as Akt in the neighboring vascular endothelial cells were detected with NaHS treatment (678).

B. Apoptosis

Apoptosis, also known as programmed cell death, is an actuality that participates in the development of different organs and systems. Abnormal apoptosis would also contribute to an array of pathological situations. Both pro- and anti-apoptotic effects of H_2S have been reported.

Inhibition of apoptosis of neurons by H_2S offers a mechanism to reduce the severity of neurodegenerative diseases (623, 641). Rotenone-induced apoptosis of human-derived dopaminergic neuroblastoma cell line (SH-SY5Y) was suppressed by NaHS (247). Among the mechanisms of NaHS effect are the inhibition of p38- and c-Jun NH_2-terminal kinase (JNK)-MAPK phosphorylation, normalized Bcl-2/Bax levels, and reduced cytochrome *c* release, caspase-9/3 activation, and poly(ADP-ribose) polymerase cleavage. The same anti-apoptotic effect of NaHS has also been observed on PC12 cells, a rat cell line derived from pheochromocytoma cells (744). In these cases, the anti-apoptotic effects of NaHS are manifested mostly at concentrations lower than 300 μM. NaHS also inhibited the apoptosis of rat hippocampus neurons induced by vascular dementia (767).

The anti-apoptotic effect of H_2S was also reported in non-neuronal cells. The examples include myocardial cells (160, 571), colon cancer cells (525), and 3T3 fibroblast cells (233). H_2S promoted the survival of cultured granulocytes in a dose-dependent (EC_{50}, 0.5 mM) and time-dependent (6–24 h) manner under stress conditions, and the delayed onset of apoptosis of granulocytes in the presence of H_2S was mediated by the inhibition of capase-3 cleavage and p38 MAPK phosphorylation (517).

The preservation of mitochondrial integrity is key to the anti-apoptotic effect of H_2S as mitochondria are usually where apoptosis process is exploded. H_2S has the capability to open K_{ATP} channels in both plasma membrane and mitochondrial membrane. Selectively blockade of mitochondrial K_{ATP} (mitoK_{ATP}) channels with 5-hydroxydecanoate has been shown to attenuate the protective effects of NaHS against rotenone-induced apoptosis of neuroblastoma cells (247). The opening of mitochondrial K_{ATP} channels will buffer the mitochondrial membrane potential ($\Delta\Psi_m$) dissipation and maintain the electrochemical gradient across mitochondrial inner membrane. These changes will affect the functionality of mitochondrial electron transport chain, mitochondrial membrane permeability, release of cytochrome *c* from mitochondria to cytosol, and the activation of caspase cascades.

A pro-apoptotic effect of H_2S on vascular SMCs was first reported in 2004. Not only NaHS but also endogenously produced H_2S stimulates apoptosis of human aortic SMCs (735). This effect was due to sequential activation of ERK and caspase-3 signaling pathways. Apoptosis of rat aortic SMCs was induced by *S*-diclofenac, a novel H_2S donor, in a dose-dependent manner (10–100 μM). This pro-apoptotic effect of H_2S was considered stemming from stabalization of p53 which subsequently induced p21, p53AIP1, and Bax (31). Later studies on other types of SMCs, such as pulmo-

nary artery SMCs, confirmed the pro-apoptotic effect of H_2S where the upregulation of Fas and caspase-3 and downregulation of bcl-2 were observed (366).

H_2S also induces apoptosis of epithelial cells. H_2S incubation (100 ng/ml) of human gingival epithelial cells caused significant DNA fragmentation (263). H_2S treatment (5, 10, and 20 ng/ml) of human gingival carcinoma cells (Ca9–22) significantly decreased DNA synthesis and decreased the proportion of cells in G_2/M phase (615). H_2S at 500 μM also induced the DNA damage of nontransformed human intestinal epithelial cells (FHs 74 Int). Four hours after H_2S treatment, COX-2 expression increased by eightfold and WNT2 was downregulated by sevenfold. At this concentration, there is no doubt that the pro-apoptotic effect of H_2S represents a genotoxic insult to the colonic epithelium. However, it is not abnormal to have this concentration of H_2S in the large intestine under physiological conditions as well as in chronic disorders such as ulcerative colitis and colorectal cancer (17).

The same effect of H_2S was seen with isolated mouse pancreatic acini (94). Application of NaHS at physiologically relevant low concentrations to pancreatic acini resulted in both early indication of apoptosis (annexin V binding) and increased activities of caspase-3, -8, and -9. And mitochondrial integrity was compromised by H_2S treatment as mitochondrial membrane potential dissipated and cytochrome *c* was released from the mitochondria. The expression of pro-apoptotic protein Bax was upregulated, but the activities of anti-apoptotic proteins Bcl-XL and Bcl-2 were unchanged (94).

Is the concentration of H_2S or its donors the determining factor for an anti-apoptotic or pro-apoptotic outcome? Indeed, the anti-apoptotic effect was observed with H_2S at 0.1–1 mM on human colon cancer HCT116 cells (525) or with NaHS at 0.01–1 mM on HUVECs (233). While NaHS at concentrations lower than 5 mM increased cell proliferation of nontransformed rat intestinal epithelial cells (IEC-18), the highest concentration of NaHS (5 mM) caused apoptosis of these cells (141). Apoptosis of gingival epithelial cells may play an important role in the onset and progress of periodontitis. Incubation of human gingival fibroblasts (HGF) and keratinocyte-like Ca9–22 cells derived from human gingival with H_2S (100 ng/ml) in the air containing 5% CO_2 led to significant apoptosis (419). This concentration of H_2S in the air, albeit high, is possible in the gingival sulcus. As such, H_2S-induced apoptosis of gingival epithelial cells and HGF may occur in the oral cavity, leading to peridontitis (419). As well, Mirandola et al. (404) found that exogenous H_2S (0.2–4 mM) induced a capase-independent cell death of peripheral blood lymphocytes that depends on their intracellular glutiathone levels. NaHS also increased lipopolysachride-induced polymorphonuclear cell apoptosis (252).

However, apoptosis can also be induced by H_2S donors at physiologically relevant concentrations or by endogenous H_2S (94, 735). NaHS at 0.2–1 mM also induced the apoptosis of mature cortical neuron apoptosis via the activation of calpain proteases and lysosomal destabilization (113).

Are differential responses of the same signaling pathways responsible for anti- or pro-apoptotic effect of H_2S or are different signaling pathways engaged? Our current knowledge is limited to address these possibilities. What we know is that, for example, H_2S activates MAPK pathway in human SMCs to induce apoptosis (735), but it inhibits MAPK pathway to limit the apoptosis of neurons (247).

With these puzzling possibilities, one take-home message could be that the opposite effects of H_2S on apoptosis are cell type-specific. As far as the mechanisms underlying this cell type-specific phenomenon are concerned, no answer is available yet.

C. Asthma and Other Respiratory Diseases

Asthma is a chronic inflammatory disease with hyperresponsive bronchoconstriction and airway remodeling, leading to extensive airway narrowing. Subepithelial fibrosis, hypertrophy, and hyperplasia of airway smooth muscle are fingerprints of airway remodeling, all leading to narrowing of the airways (32, 680). The mechanism of airway remodeling is not fully understood.

H_2S can be produced in the lung and airway tissues via the actions of CSE and CBS. CSE protein has been detected in the airway and vascular smooth muscle cells in rat peripheral lung tissues using immunohistochemical staining (111). CBS mRNA is also detected in rat lung tissues (364). Distribution of CSE and CBS to airway SMCs, vascular SMCs, and vascular endothelial cells in the lung of mice has also been shown with immunohistochemical staining (680). Therefore, endogenously generated H_2S may participate in the regulation of contractility of airway smooth muscles and lung circulaiton.

It has been suggested that decreased CSE expression and H_2S levels in pulmonary tissues are related to the pathogenesis of asthma (109). Ovalbumin (OVA) challenge is the most often used animal model in asthma study. After treatment of rats with OVA, the rats developed asthma and H_2S levels in serum and lung tissues were significantly decreased (109, 364). The expression of CSE protein in lung tissues (Western blot) was also decreased by 78% in OVA-treated rats versus in control rats. There is a positive correlation between H_2S levels in serum and lung tissue with peak expiratory flow (PEF) and a negative correlation with the proportion eosinophils and neutrophils in bronchoalveolar lavage fluid (BALF), scores for inflammatory cell infiltration, collagen deposition, and goblet cell hyperplasia (109).

Administration of NaHS via intraperitoneal injection to these rats reduced their airway inflammation and airway remodeling as revealed by BALF cell counts, expression of inflammatory genes, and airway histology analysis (109). NaHS treatment also increased PEF, indicating alleviation of airway obstruction (109). Interestingly, NaHS treatment significantly attenuated the activation of pulmonary iNOS in OVA-treated rats, whereas iNOS expression was not altered. A molecule-to-molecule interaction between H_2S and iNOS may occur and the anti-asthma effect of H_2S may be partially realized by its inhibitory action on iNOS activity. This hypothesis can be relatively easily tested by altering iNOS expression and activity and then applying H_2S to OVA-animal models. Another study showed decreased CBS mRNA expression, together with the decreased CSE mRNA expression, in OVA-challenged rats (364). The relative contribution of CSE and CBS to the decreased production of H_2S in asthmatic lungs should be further examined. The correlation of CSE/CBS/H_2S with hyperresponsiveness of bronchoconstriction is another item that requires clarification. Intraperitoneal injection of NaHS will increase H_2S level in whole body, but selective administration of NaHS into the lung has not been employed.

The metabolism of endogenous H_2S in patients with asthma has been investigated as well. The levels of serum H_2S, lung function, and cell differential count in induced sputum were studied in 44 patients with acute exacerbation of asthma, 33 patients with stable asthma, and 12 healthy subjects. The serum H_2S level was around 75 μM in health subjects, 56 μM in patients with stable asthma, 58 μM in patients with mild acute exacerbation asthma, and 41 to 31 μM in moderate to severe acute exacerbation asthma. The severity of asthma is proportional to the decrease in H_2S level and negatively correlated with the count of sputum cells and the percentage of sputum neutrophils (718). Whether altered endogenous H_2S level can be the cause or consequence of airway obstruction in asthma is not clear. It has been suggested to use endogenous H_2S level as a noninvasive marker of asthma activity and severity. Decreased H_2S production in the lung may lead to lower level of H_2S in exhaled or nasal air, and as such, it may serve as an early diagnosis biomarker for asthma. Of course this realization has to be built up on the premises that decreased lung production of H_2S is closely correlated with pathogenesis progress of asthma and the H_2S detection method is sensitive enough to catch the H_2S in exhaled or nasal air (680).

Oleic acid (OA)-induced acute lung injury (ALI) represents another experimental model for respiratory diseases. The treated animals specifically showed decreased partial pressure of oxygen in the arterial blood (Pa_{O_2}) levels, an increased pulmonary wet/dry weight (W/D) ratio, increased index of quantitative assessment (IQA) score, and increased frequency of polymorphonuclear (PMN) cells in the lung 2, 4, or 6 h after OA injection (0.1 ml/kg iv) (365). These animals also had decreased H_2S levels and increased IL-6, IL-8, and IL-10 levels in the plasma and lung tissue. NaHS treatment (56 μM ip) of OA-treated rats reversed all ALI-related pathologies. NaHS also decreased IL-6 and IL-8 levels in the plasma and lung tissues. These observations may help to understand the correlation of endogenous H_2S metabolism and the occurrence of ALI. The corresponding therapeutic strategies may also be better devised.

Chronic obstructive pulmonary disease (COPD) is another human respiratory ailment, with airway inflammation playing a role in its pathogenesis. Altered metabolism of endogenous H_2S may be involved in the pathogenesis of airway inflammation and airflow obstruction in COPD. Chen et al. (110) showed that serum H_2S level was increased in patients with stable COPD than in other populations. Among stable COPD patients, H_2S levels differed based on the stage of airway obstruction, lower in patients with stage III than stage I obstruction. Serum H_2S level was lower in patients with in patients with acute exacerbation of COPD (AECOPD). In another clinical study of 129 patients and 72 healthy control subjects, the mean serum H_2S concentration was 36% lower in patients with pneumonia (23 μM) than in control subjects (35 μM). However, serum H_2S concentration did not differ between patients with acute exacerbations of COPD (34 μM) and control subjects. This result was in conflict with the previously reported decrease in H_2S level with AECOPD (110).

Further studies tried to elucidate whether endogenous H_2S mediates the anti-inflammatory effect of short-term theophylline treatment on airway inflammation in 37 patients with stable COPD (111). Theophylline is a nonselective phosphodiesterase inhibitor that has bronchodilator/anti-inflammatory properties and is widely used in the treatment of airways diseases. Although short-term theophylline treatment improved symptoms and decreased sputum neutrophils in COPD, the studied patients did not show altered serum H_2S levels. However, a conclusion cannot be made yet on the role of H_2S in this COPD treatment due to the rather small group of patients recruited and due to the short treatment regime.

D. Atherosclerosis

Atherosclerosis is a chronic, systemic disease with multiple factors involved in its initiation and progression. It adversely affects the structure of blood vessels. Vascular inflammation, endothelial damage, smooth muscle cell migration, foam cell accumulation, and lipid and cholesterol deposition contribute to different stages of plaque formation in large and medium-sized blood vessels. The consequential narrowing and stiffening of blood vessels restricts blood circulation and increases plaque thrombogenicity. Atherosclerosis-associated cardiovascular disease is the leading cause of death in the developed nations and is increasing

rapidly in developing countries. Pathogenic causes that lead to these pathological changes in atherosclerosis have always been the center of attention, and the preventive as well as therapeutic strategies have always been pursued. H_2S may be involved in both the pathogenesis and the treatment of atherosclerosis.

One of the major events in pathogenesis of atherosclerosis is the proliferation and/or apoptosis of vascular SMCs. After inhibiting endogenous H_2S production with PPG or knocking down endogenous CSE gene by short interfering RNA approach, H_2S at 50–100 μM induces apoptosis of human aorta SMCs (735). Without inhibiting endogenous H_2S production, exogenously applied H_2S at 100 μM had little effect on SMC apoptosis. The importance of the interaction between exogenous and endogenous H_2S is thus indicated. In cultured human aorta SMCs, H_2S activated ERK and caspase-3 (735). After inhibiting ERK and caspase-3, the apoptosis of human aorta SMCs induced by H_2S was significantly attenuated. Promoting endogenous production of H_2S by overexpression of CSE in human aorta SMCs also inhibited cell growth and induced apoptosis (739). The deposition of oxidized LDL in the endothelium would stimulate the proliferation of vascular SMCs. The atherogenic modification of LDL induced by HOCl (the product of the activated myeloperoxidase/H_2O_2/chloride system) was inhibited by NaHS (334). This could be related to the scavenging effect of H_2S on HOCl as well as the interaction of H_2S with the substrate, MPO and H_2O_2, of the myeloperoxidase/H_2O_2/chloride system.

H_2S reduces atherogenesis. H_2S inhibited atherogenic modification of purified LDL induced by hypochlorite in vitro, as measured by apolipoprotein alterations (334). Meng et al. (400) reported that during neointimal formation induced by balloon injury in rats, CSE expression was reduced and endogenous production of H_2S decreased. Rescue of the injured artery with NaHS injection not only reversed the reduced endothelium-dependent vasorelaxation, but also significantly inhibited neointima formation of the balloon injured carotid arteries.

Homocysteine is a key amino acid in regulating cellular levels of cysteine, methionine, and sulfur. Accumulation of homocysteine in the plasma, termed hyperhomocysteinemia (HHcy), is an independent and graded risk factor for atherosclerosis and atherosclerosis-related cardiovascular disease. Homocysteine may induce vascular damage by promoting platelet activation, hypercoagulability and thrombosis formation, oxidative stress and activation of proinflammatory factors, endothelial dysfunction, vascular SMC proliferation, and ER stress (18, 19). Animal studies have demonstrated that HHcy enhances vascular neointima formation and accelerates atherosclerosis (411). However, the underlying mechanisms for the involvement of homocysteine in the pathogenesis of atherosclerosis are still not fully understood.

Deficiency of either CBS or CSE leads to HHcy. CSE KO mice have significantly higher homocysteine levels in their plasma than that of wide-type mice (738). Low levels of NaHS (30 or 50 μM) protected rat aortic SMCs from homocysteine-induced cytoxicity and reactive oxygen species, leading to improved cell viability (730). H_2S (30 μM) attenuated the homocysteine (100 μM)-induced overproduction of oxidized DCF (reflecting the levels of H_2O_2 and $ONOO^-$) and O_2^- by 13.8 and 55.8%, respectively (730). It was thus concluded that H_2S played a protective role on HHcy-induced cell injury due to its antioxidative stress effect. In addition to interacting on the common targets, H_2S level directly impacts on the plasma homocysteine level. In one experiment, homocysteine solution was administered to rats subcutaneously twice a day at 8-h intervals from day 1 to 21. The plasma homocysteine level of the treated rats was comparable to that of patients with moderate HHcy levels (~36 μM). Injection of the same animals with NaHS at 2.8 or 14 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ (ip) lowered plasma homocysteine concentrations to 24 or 18 μM, respectively. HHcy-induced lipid peroxidation formation was also suppressed by NaHS treatment (98).

Dietary supplementation with methionine and homocysteine induces HHcy, which promotes early atherosclerosis in apolipoprotein E knockout (apoE KO) mice (777). CBS or CSE deficiency also leads to HHcy. Can HHcy and lack of H_2S produce a compound effect on atherosclerosis development? A double-knockout mouse for both ApoE and CBS was created for answering this question by breeding $CBS^{-/+}$ females with $apoE^{-/-}$ males. It should be noticed that much of the results were obtained from $CBS^{-/+}$/$apoE^{-/-}$ mice. The true double knockout mice ($CBS^{-/-}$/$apoE^{-/-}$) struggled to survive during the first 3 postnatal weeks. And "about 5% of $CBS^{-/-}$/$apoE^{-/-}$ survived to 15 wk of age, about 2% to 6 mo" (675). Meanwhile, the atherosclerotic lesions would manifest themselves significantly only after 15 wk to 6 mo. Therefore, the contribution of CBS, mostly being heterozygous knockout, to the pathological process should be comprehended with this in mind. Furthermore, endogenous level of H_2S and CSE expression level (reflecting a potential compensatory change) were not determined in this study. Nevertheless, ApoE/CBS KO mice developed aortic lesions and HHCy in an age-dependent way without dietary manipulation. Lesion area and lesion cholesteryl ester (CE) and triglyceride (TG) contents increased, but cholesterol esterification and activities of enzymes catalyzing CE or TG formation in the vessel wall and in peritoneal macrophages were not changed (675). It was concluded that HHcy accelerates atherosclerosis in ApoE/CBS KO mice. To date, the role of CSE and CSE-produced endogenous H_2S in the pathogenesis of atherosclerosis have not been directed examined.

Atherosclerosis model of rats has also been used to study the role of H_2S. Male SD rats fed with high grease food and injected with vitamin D_3 developed atherosclerosis with significantly increased serum TG and cholesterol. Their scores of the artery pathological damage increased from week 6 to week 12 when atherosclerotic plaque appeared (108). H_2S level in serum bore no correlation with the levels of TG and cholesterol but was negatively correlated with both pathological damage scores and the expression of VEGF (108). The meaning of this correlation analysis is limited, since it would not tell whether the altered H_2S metabolism is causative for atherosclerosis or a parallel disturbance due to the special diet treatment.

H_2S would also alter the progress of atherosclerosis by interfering with vascular inflammation. H_2S attenuated LPS-induced inflammation in cultured microglia (248). H_2S-inhibited leukocyte infiltration and adhesion have also been reported (755). Calcification is another significant risk factor of atherosclerosis. It escalates the risk of myocardial infarction and aggravates plaque instability. Vitamin D_3 plus nicotine treatment is an established animal model for vascular calcification. In the treated rats, calcium content and osteopontin mRNA in calcified arteries were significantly elevated. CSE activity and expression levels were downregulated together with decreased H_2S content in the calcified aorta. Supplementation of NaHS to the treated rats lowered vascular calcium content and osteopontin mRNA. How does H_2S interfere with vascular calcification? One theory is that the oxidative stress level is lowered by H_2S (719).

Direct management of artherosclerosis in living animals was realized in a 2009 study. Treatment of apoE KO mice with NaHS resulted in reduced atherosclerotic plaque, whereas inhibition of CSE activity in apoE KO mice with PPG enlarged plaque size. The effect of NaHS is linked to ICAM-1 since this treatment reduced ICAM-1 level in circulation and its expression in aortic endothelial cells (690). By promoting adhesion of inflammatory cells to the endothelium, ICAM-1 may be one of the causative factors for atherosclerosis. In fact, its level has been shown to be significantly higher in human atherosclerotic plaques. This exciting discovery certainly invites further investigation. The most intriguing question so arose, however, is whether abnormality of CSE/H_2S system is the cause or consequence of the disease. The promising side of the study is that H_2S level in apoE KO mouse plasma was marginally lower, which was linked to advanced atherosclerosis. The not-so-clear side is that basal levels of H_2S and CSE protein expression before the manifestation of atherosclerosis in apoE KO mice were not determined. The aortic production rate of H_2S and aortic CSE expression level in apoE KO mice were even more perplexing. Decreased H_2S production rate should reflect decreased CSE activity or expression. The decreased H_2S production rate as reported (690), however, contradicts with the increased CSE mRNA expression in aortic atherosclerotic tissues, which cannot be readily explained by an assumed "positive-feedback" mechanism. Furthermore, the existence of this positive-feedback mechanism between H_2S and CSE expression would also challenge the clinical application of exogenous H_2S therapy to atherosclerotic patients as it would inhibit CSE expression and decrease endogenous H_2S level. This would not sound like good news for the prognosis of atherosclerotic patients (685).

E. Cancer

H_2S has been known for its anti-apoptotic and pro-apoptotic effects (see previous discussions). When it comes to cancer, the dominant side of the double-edge effects of H_2S will determine the outcome of cancer. WiDr cells are from a colon epithelial cancer cell line. These cells express both CBS and CSE. Cao et al. (93) treated these cells for 24 h with butyrate, a short-chain fatty acid that arrests growth of a variety of cells, and found that both cell production of H_2S and the expression of CBS and CSE were increased. Furthermore, butyrate treatment killed the cancer cells and so did NaHS (50–200 μM). Now the question is whether the anti-cancer effect of butyrate is mediated by endogenous H_2S. In this regard, blockage of CBS activity by AOA also partially but significantly inhibited butyrate-induced cell death. NaHS and butyrate have different signaling targets. Although NaHS treatment stimulated the phosphorylation of ERK and p38 MAPK, the inhibition of both did not affect butyrate-reduced cell viability. Butyrate had no effect on p38 MAPK activation. Interestingly, the suppression of p38 MAPK activation further strengthened, rather than inhibited, NaHS-reduced cell viability (93). Thus butyrate and H_2S shared the same anti-cancer properties, but their molecular targets are not exactly matching. These observations may have a wide impact on the study of colon cancer biology and the prevention of colon cancer and chronic intestinal epithelial disorders. In contrast to the report by Cao et al. (93), NaHS (20 μM) promoted the proliferation of another colon cancer cell line, HCT 116 cells (87). Increased Akt and ERK phosphorylation, decreased activity of cyclin-dependent kinase inhibitor p21(Waf1/Cip1), and altered NO metabolism were attributed to this pro-proliferation effect of NaHS. The reason for these conflict reports is not clear. On the other hand, indirect evidence had been provided for the anti-cancer effect of H_2S against colon cancer. The progress of advanced colon cancer is correlated with decreased expression of TST, but H_2S (100 μM) increased TST activity in HT-29 cells, a human colon cancer cell line (506). Thus it can be reasoned that sulfide-increased TST activity would be anti-cancer in nature. Butyrate also increased TST activity and expression, similar to the effect of sulfide, in HT-29 cells (506). This observation is in line with the reported anti-cancer effects of both butyrate and H_2S (93).

F. Colitis

As discussed earlier, bacteria represent a unique source of H_2S production in the GI tract. Certainly, H_2S from this source should be considered for its impact on ulcerative colitis. At the concentrations commonly found in the lumen of human colon, H_2S impaired the utilization of butyrate by colonocytes (522). As such, H_2S stimulated epithelial proliferation (114) and increased epithelial permeability (437). The luminal concentration of H_2S is significantly higher in patients with ulcerative colitis than healthy people (487), and the H_2S production rate is correlated with the severity of colitis (184). Levine et al. (353) collected fecal samples from 25 subjects with ulcerative colitis and 17 controls and measured the gas release from these samples at intervals over 24 h. A three- to fourfold increase in H_2S release by ulcerative colitis feces was detected at every measurement point compared with normal feces (353). This fecal effluent analysis and many others (487, 530) associate an increase in bacteria-produced H_2S with colitis. Does this association represent a causative relationship so that increased H_2S level intoxicates or damages colon tissues?

Feeding of rats with nonabsorbable, carbohydrate-bound sulfate in the form of dextran sulfate or carrageenan produces a model of colitis. The development of this rat colitis was blocked by metronidazole, pointing to the pathogenic role of a sulfur-containing compound which might be released during the bacterial metabolism of the nonabsorbed sulfate. Feeding rats with dextran sulfate increased fecal release of H_2S, but in the presence of bismuth subsalicylate, a compound that avidly binds H_2S, this increased release of H_2S was reversed (196). However, excessive H_2S production does not suffice to justify a pathological role of H_2S for colitis (196). Results derived from rectal biopsies and real-time polymerase chain reaction showed no disease-related difference in sulfate-reducing bacteria carriage between patients with ulcerative colitis and controls (179). Studies on ulcerative colitis and Crohn's disease also did not find deficiency of enzymic detoxication of sulfide by rhodanese and thiol methyltransferase in rectal mucosa and erythrocytes, and plasma thiocyanate (485). These studies conclude that increased H_2S production from sulfate-reducing bacteria is not the cause of colitis.

On the other hand, the anti-inflammatory effect of H_2S would provide important mucosal defense and thus promote ulcer healing and suppress colitis. This protective role of H_2S has been shown in both mouse and rat models of colitis. In a rat model of colitis induced by intracolonic administration of trinitrobenzene sulfonic acid, Wallace et al. (672) found that H_2S synthesis is markedly upregulated over the first days after the induction of colitis and then declined toward control levels as the colitis was resolved. Inhibition of H_2S synthesis interfered with resolution/healing of the colitis and the animals became fatal, while administration of H_2S donors intracolonically accelerated resolution/healing. The protective effect of H_2S is not only restricted to rats with colitis. Inhibition of H_2S synthesis also led to mucosal injury in the small intestine and colon from normal control rats.

ATB-429, a H_2S-releasing mesalamine derivative, reduces colitis-associated leukocyte infiltration and the expression of pro-inflammatory cytokines in the mouse colon (181). Hirata et al. (239) treated male BALB/c mice with 8% dextran sodium sulfate (DSS) to induce acute colitis. The animals experienced weight loss, stool consistency, and intestinal bleeding, which were exacerbated by PPG treatment. Myeloperoxidase activity and thiobarbituric acid-reactive substances in the inflamed mucosa were also increased by PPG. These results suggest that endogenous H_2S protects the mucosa from inflammatory injury. Reduced endogenous production of CSE-based H_2S by PPG treatment could explain the deterioration of colitis, which was further supported by the observation that Na_2S treatment cancelled off the effects of PPG.

ATB-429 also inhibited nociception to colorectal distension (CRD), a rat model that mimics some features of the irritable bowel syndrome (147). Similar results were obtained with NaHS treatment. The effects of these two H_2S donors appear to be mediated by the activation of K_{ATP} channels or NO synthesis. On the other hand, increasing endogenous H_2S production by administering the substrate of CSE, L-cysteine, reduced rectal sensitivity to CRD.

In summary, we have a very unique situation here. The colon tissues have been exposed to high level of H_2S in colitis due to increased activities of sulfate-reducing bacteria, which seems not being a pathogenic factor for colitis. On top of this already higher H_2S level, further increase in colon tissue production of H_2S or supplement of exogenous H_2S donor actually suppresses the development of colitis. The questions are therefore posted. Why is the high level of bacteria-produced H_2S not enough for colon protection and what level of H_2S is high enough for the same? What is the difference between bacteria-produced H_2S and colon tissue produced H_2S in terms of protection against colitis?

G. Dermal Wound Healing

Dermal wound healing is a complex process composed of early reactive inflammation, re-epithelialization, granulation, and tissue remodeling. A speeded dermal wound healing process has been attributed to H_2S. Application of NaHS to the wounded skin enhanced wound healing in a rat burn model (30% of the total body surface area dorsal scald burn). Wound closure after 1 mo was markedly improved in animals receiving daily topical administrations of H_2S (467). Using the same rat model, an earlier study used subcutaneous injection with IK-1001, a H_2S donor, to treat the burned animals, which was started 48 h post burn and

continued for 4 wk. Reepithelialization was improved by IK-1001 treatment significantly.

The role of endogenous H_2S in dermal wound healing could not be firmly established until a study showed that the knockout of CSE significantly delayed the healing process in the genetically engineered mouse (467). In this study, 16-wk-old male mice carried an ~100-mm^2 scald wound (5% total body-surface area) on their dorsal surface. Throughout the observation period of 21 days, wound areas in CSE KO mice were consistently smaller than in wild-type mice, and the wound healing was complete in wild-type mice at least 5 days earlier than in CSE KO mice. These observations demonstrate that endogenous H_2S is a pro-healing factor.

H. Diabetes

Altered insulin metabolism and reduced insulin sensitivity are two trademarks of diabetes mellitus. Albeit with concentrated attention being paid to different circulating insulin levels in diabetes, not so much has been known about altered insulin release from pancreas in diabetes and the underlying mechanisms. Insulin release from pancreatic beta-cells is tightly coupled to glucose metabolism. In addition to an amplifying pathway putatively involving mitochondrial production of glutamate, an extracellular calcium entry-dependent mechanism controlled by K_{ATP} channels in beta-cell membrane is a pivotal event in glucose-regulated insulin release cascade. In this event, increase in circulating glucose results in production of ATP from beta-cell mitochondria. A high ATP/ADP ratio subsequently closes K_{ATP} channels in pancreatic beta-cells, leading to membrane depolarization. Resultant opening of voltage-dependent calcium channels injects more calcium into the cytosolic milieu. This eventually leads to exocytosis of insulin granules.

If endogenous H_2S level was elevated in pancreatic beta-cells, insulin release would be reduced and hyperglycemia would occur. This hypothesis was firstly tested on Zucker diabetic fatty (ZDF) rats by Jia et al. in 2004 (278). ZDF rats represent a genetic animal model of type 2 diabetes mellitus. These rats are characterized with hyperglycemia and peripheral insulin resistance. CSE gene expression is abnormally higher and so is H_2S production in pancreatic beta-cells of ZDF rats than those of nondiabetic Zucker lean or Zucker fatty rats (278). This observation was later confirmed by Wu et al. (717). Compared with the expression level of CSE, the expression level of CBS mRNA in rat pancreatic islets is extremely low. Pancreatic islet H_2S production is predominantly regulated by CSE, rather than CBS (278, 717). Inhibition of CSE activity by PPG significantly decreased production of H_2S, increased plasma insulin level, and lowered hyperglycemia in ZDF rats (278, 717). The upregulation of CSE/H_2S pathway precedes the appearance of hyperglycemia, indicating that the elevated CSE/H_2S level is not initially triggered by hyperglycemia (717). In fact, hyperglycemia may inhibit CSE/H_2S pathway in pancreas to form a feedback loop for fine regulation of glucose-induced insulin secretion. It is known that H_2S production in INS-1E cells was inhibited by hyperglycemia (741). Zhang et al. (766) showed that high glucose (20 mM) incubation of INS-1E cells or freshly isolated rat pancreatic islets inhibited CSE expression, CSE activity, and H_2S production. One of the underlying mechanisms is the hyperglycemia-induced phosphorylation of Sp1, which may subsequently stimulate p38 MAPK. Whether the phosphorylation of Sp1 alters the Sp1 binding to the CSE promoter region was not studied (766).

Increased pancreatic H_2S level would impact on insulin release and function in four different ways. First, it stimulates whole cell K_{ATP} channels to hyperpolarized beta cells. This was initially demonstrated in cloned insulin-secreting beta cell line, INS-1E cells, by recording whole cell and single-channel K_{ATP} channel currents (741). Increase in extracellular glucose concentration significantly decreased endogenous production of H_2S in INS-1E cells and increased insulin secretion. After transfection of INS-1E cells with adenovirus containing the CSE gene (Ad-CSE) to overexpress CSE, high glucose-stimulated insulin secretion was virtually abolished. Basal K_{ATP} channel currents were significantly reduced after incubating INS-1E cells with a high glucose concentration (16 mM) or lowering endogenous H_2S level by CSE-siRNA transfection. Under these conditions, exogenously applied H_2S significantly increased whole cell K_{ATP} channel currents at concentrations equal to or lower than 100 μM. H_2S (100 μM) markedly increased open probability by more than twofold of single K_{ATP} channels (inside-out recording) in native INS-1E cells. Single-channel conductance and ATP sensitivity of K_{ATP} channels were not changed by H_2S. H_2S stimulates K_{ATP} channels in INS-1E cells independent of activation of cytosolic second messengers. The stimulatory effect of H_2S on K_{ATP} channels has also been shown in freshly isolated rat beta cells (717). The induced membrane hyperpolarization by H_2S will decrease calcium entry and insulin release.

Second, diabetes is a spectrum of clinical conditions that arose from relative or absolute insulin deficiency with decreased functional beta cell mass. Any change in beta-cell mass must reflect a misbalance between proliferation (neogenesis or replication) and cell death (necrosis or apoptosis) (540). Excessive loss of beta cells constitutes one of the causes of diabetes, and apoptosis is considered to be the main mode of beta-cell death in the pathogenesis of type 1 diabetes and progression of type 2 diabetes (374, 444). Given the importance of pancreatic beta-cell mass for the pathogenesis of diabetes and altered endogenous pancreatic production of H_2S in diabetes, a reduced beta-cell mass by H_2S would also be important to the pathogenesis of diabetes. The beta cell-killing effect of H_2S is especially relevant

for type 1 diabetes. H_2S-induced apoptosis of INS-1E cells has been shown, which was mediated by enhancing ER stress via p38 MAPK activation (740). However, another study showed that L-cysteine or NaHS suppressed islet cell apoptosis with high glucose and increased glutathione content in MIN6 β-cells (295). While it is well known that STZ causes diabetes in animal models by destroying pancreatic beta-cells, eliminating CSE gene expression renders the animal more resistant to STZ attack. Yang et al. (736) recently showed that CSE-KO and WT mice had matchable blood glucose and plasma insulin levels. Approximately 5 days after injection with STZ, hyperglymecia developed in WT mice, but it was not manifested until about 15 days later in CSE KO mice. One month after STZ injection, blood glucose level reached 28 mM in WT mice, but it was only 14 mM in CSE mice. The interaction of STZ and CSE activity was further substantiated as the inhibition of CSE activity in WT mice by PPG treatment for 30 days partially suppressed STZ-induced hyperglycemia and hypoinsulinemia. More apoptotic β-cell death was observed in wild-type mice than in CSE KO mice after STZ treatment. This in vivo phenomenon was confirmed with the cultured INS-1E cells whose viability was significantly reduced by the inclusion of STZ in the culture medium. The researchers therefore reasoned that STZ might destroy beta-cells by acting on CSE/H_2S pathway. Indeed, pancreatic H_2S production was increased after STZ treatment in WT mice. This increase was due to the stimulation of CSE since STZ treatment of CSE KO mice yielded no change in pancreatic H_2S production. Additional evidence for the above notion was derived from the STZ-enhanced H_2S production in cultured INS-1E cells (736).

Third, insulin also exerts an autocrine stimulation for insulin release from pancreatic beta-cells. By acting on insulin receptor substract-1, extracellular insulin increases $[Ca^{2+}]_i$ and promotes insulin release from beta-cells (16). Suppression of this autocrine pathway of insulin significantly reduced islet size and beta-cell mass and resulted in defected insulin release and development of insulin resistance (330). Insulin receptors on pancreatic beta cells may be modified by H_2S so that insulin-regulated insulin release might be altered. Injection of the mice with NaHS has been shown to lead to an instant increase in blood glucose, decreased plasma insulin, and deteriorated glucose tolerance in mice (736).

Fourth, H_2S may directly interact with insulin molecule to modify its structure and function. The consequently formed H_2S-insulin adducts would not be recognized by either its intracellular transport vesicle for releasing or by the insulin receptor on the external surface of beta cell membrane. Since there are three disulfide bonds in insulin molecule, the formation of H_2S-insulin adduct at one or several cysteine residues can be speculated. In this regard, the molecule-to-molecule interaction between H_2S and methylglyoxal would be informative. Chang et al. (99) found that a direct molecule-to-molecule reaction methylglyoxa and H_2S changed the structure and function of both the involved molecules.

Wu et al. (717) thus proposed a "sweetened rotten egg" model to elucidate physiological regulation of pancreatic function. This model integrates the role of pancreas in regulation of "sweet" glucose metabolism and inhibitory role of the "rotten egg" gas H_2S in insulin release (717). As abnormal insulin release is ubiquitous for many disorders of insulin resistance syndrome, including obesity and hypertension, a critical evaluation of the role of pancreas as a sweetened rotten egg will shed light on understanding of pathogenesis and management of these disorders. As a support for this hypothesis, within hours of administration of H_2S into the animals, increased glucose level and decreased insulin level were evident. These effects were all blocked by the administration of glibenclamide (473). In STZ-induced diabetic animals or ZDF rats, hepatic or pancreatic activities of CBS and CSE were also significantly upregulated associated with decreased plasma homocysteine level (507, 711, 754). Moreover, a study of the effect of H_2S in protecting the heart from I/R indirect supports the pathogenic role of H_2S in diabetes. Calvert et al. (90) found Na_2S (100 μg/kg) administration (intravenous) at the time of reperfusion reduced myocardial area-at-risk (AAR) per left ventricle (LV) and infarct size per AAR (INF/AAR) by 56% in nondiabetic mice. In contrast, diabetic mice treated with Na_2S only exhibited a 20% reduction.

The "sweetened rotten egg model" certainly requires more animal studies to validate and more importantly human studies to verify. In type 1 and type 2 diabetic patients without nephropathy, the activities of CBS and CSE were significantly enhanced compared with healthy controls (658, 754). On the other hand, comparison of blood levels of H_2S revealed a two- and fourfold lower level of H_2S in metabolic syndrome patients and type 2 diabetic patients, respectively, than in lean and healthy humans. Increased insulin resistance, hypertension, and higher lipid level were also manifested in those patients with low blood H_2S levels (702). Is this observation contradictory with the "sweetened rotten egg" model of diabetes? Not necessarily. Remember what is critical in the "sweetened rotten egg" model is the H_2S metabolism in pancreatic beta cells, not in blood. Blood level of H_2S reported by Whiteman et al. (702) is affected by, and affects on, multiorgans. Pancreatic metabolism of H_2S in humans will be more revealing in this regard.

I. Erectile Dysfunction

Like hypertension or stroke, erectile dysfunction is also largely a blood vessel problem. Penile corpus cavernosum is a highly vascularized tissue whose functional status depends on an equilibrium between vasodilatory and vaso-

constrictory tone. Penile erection is maintained by the dilation of blood vessels, or the relaxation of sinusoidal smooth muscle cells in the penis. The wonder drug for treating erectile dysfunction came from the realization that penile erection is largely ascribed to a local surge of NO acting to dilate the vessels (83); thus Viagra (sildenafil) does one thing very well, which is to sustain the local effect of NO on the penis by suppressing the breakdown of cGMP.

H_2S may produce the same functional outcome as NO in dealing with erectile dysfunction. H_2S was produced in rabbit corpus cavernosum, which is the tissue responsible for penile erection. When exposed to NaHS (0.1–3.2 mM), these penile tissues in organ bath relaxed via the production of cAMP, different from the yield of cGMP after NO exposure. Inhibition of CSE with PPG or BCA and that of CBS with AOA markedly increased the noradrenergic contractile neurotransmission of corpus cavernosum strips to field stimulation. Thus the role of endogenous H_2S in relaxing penile tissues was hinted (587, 588). When NaHS was injected into the penis of tranquilized monkeys, the length of the penis increased by 8–74%, depending on the dosage of NaHS (587, 588). This is not a case of "penile growth," however, as penile length increased within minutes and returned to basal level before the next NaHS injection. In rats, pharmacological manipulation to reduce the levels of H_2S produced in the penis blunts the nerve pulses that constitute the penile reaction to sexual stimulation (136).

Does the same pro-erectile effect of H_2S apply to humans too? An earlier study already showed that human corpus cavernosum, obtained by a standardized surgical procedure, expressed both CBS and CSE (406). More specifically, peripheral nerves in penile tissues had only CSE expression, whereas the muscular trabeculae and the smooth muscle component of the penile artery had both CBS and CSE expression (136). A 2009 study reported that human penile tissues converted L-cysteine to H_2S. On the other hand, isolated human penile tissues responded to exposure to NaHS or a precursor of H_2S (L-cysteine) by relaxing, the reaction that would normally trigger an erection of the penis in vivo (136). PPG or AOAA enhanced electrical field stimulation-induced tension development of penile tissue, suggesting the involvement of both CSE and CBS. Unlike the proposed cAMP pathway proposed by the previous study on rabbits (587), H_2S-induced human erection appears to be mediated by K_{ATP} channels in penile smooth muscle cells (136).

These discoveries may have applications to many diseases where erectile dysfunction occurs, such as diabetes, hypertension, and obesity. On the other hand, the pro-erectile role of the "sour gas" has invited some "sour" questions. For one, if H_2S in the penis were important for the erection, why does knocking out the H_2S-generating enzyme CSE in mice reportedly not affect their fertility (738)? It is possible that NO is more important for the erection while H_2S, though produced in the penis, may be more relevant to something else other than the erection. CO is also produced in the penis, but this gas is involved in the facilitation of ejaculation, not erection (82). The lack of CSE in the mouse penis is likely compensated for by increased CBS, another enzyme that produces H_2S, which should be tested in CSE knockout mice. The relative contribution of CBS to penile erection is intriguing. In rats, L-cysteine-induced penile tissue relaxation is blocked by PPG (a CSE blocker), but it is AOA (a CBS blocker) that blocked the same effect of L-cysteine on human penile tissues (136). Certainly, species difference has to be considered in evaluating the role of H_2S in regulation of penile erection. Reproduction biology of humans is very different from that of mice so that we cannot directly extrapolate mice discoveries to humans. Finally, assuming that CSE/H_2S in the penis is important for maintenance of the erection, residual erection in the absence of H_2S may suffice for ejaculation to induce fertilization.

Another challenge comes down to the relative potency of NO and H_2S in treating erection dysfunction. In the aforementioned human study (136), NaHS appears not to relax penile tissues unless its concentration is increased to a level between 100 μM and 1 mM, making it much less potent than NO and warranting concerns of toxicity of these effective dosages. On the other hand, the postproduction life span of NO is much shorter than the relatively stable life span of H_2S. Therefore, the long-lasting proerectile function of H_2S may compensate for its low potency. Advance in gasotransmitter study may assist in unraveling the individual and collective roles of NO, CO, and H_2S in modulating erectile dysfunction and sexual arousal disorders.

Some pharmaceutical companies are already comparing H_2S donors with Viagra-like compounds. Don't be surprised if one soon finds a H_2S-based "Viagra" available in the nearest pharmacy. In fact, the antierectile efficacy of a H_2S-donating derivative of sildenafil (ACS6) has been compared with sildenafil citrate and NaHS on isolated rabbit cavernosal tissues and rat smooth muscles (560). ACS6 and sildenafil citrate relaxed cavernosal smooth muscle equipotently. The effects of both compounds are exclusively due to the sildenafil component, since NaHS alone had little effect at up to 100 μM. Interestingly, ACS6 had the greatest potency in inhibiting the formation of superoxide and suppressing type 5 phosphodiesterase (PDE5), followed by sildenafil citrate and then NaHS (560).

J. Hypertension

1. Essential Hypertension

The vasorelaxant property of H_2S gives this gasotransmitter a role in regulating peripheral resistance and blood pressure. Reduced endogenous H_2S level, especially in blood

vessel wall, would be in favor of hypertension development, logically. This was indicated in an earlier study which showed that administration of the CSE inhibitor PPG elevates blood pressure in rats (771). Direct evidence for the role of endogenous H_2S in blood pressure control came later when a CSE gene deficient mouse was generated (738). The researchers showed that CSE knockout mice manifested with age-dependent development of hypertension at the level comparable to that of eNOS KO mice (337). The elevated blood pressure in CSE KO mice was not related to the contractile status of peripheral blood vessels since phenylephrine-induced constriction of isolated mesenteric arteries was not different between wide-type and CSE KO mice. On the other hand, endothelium-dependent vasorelaxation, triggered by the activation of muscarinic cholinergic receptors on the endothelium, was severely abolished in CSE KO mice. Therefore, it was concluded that hypertension in CSE KO mice is caused by the elimination of CSE expression in vascular tissues, especially in the endothelium, and the loss of H_2S as a critical EDRF in regulating peripheral resistance.

The role of H_2S in the pathogenesis of spontaneously hypertensive rats (SHR) was also examined. SHR develop hypertension spontaneously, coincident with decreased H_2S production and CSE expression in aortic tissues and lowered plasma level of H_2S (729, 774). Treatment of SHR with NaHS for 5 wk suppressed hypertension development, and partly reversed the hypertension-related vascular remodeling and collage accumulation, i.e., lowering hydroxyproline and collagen type I levels in aortic tissues. NaHS incubation also inhibited angiotentin II-stimulated [^{3}H]TdR and [^{3}H]proline incorporation as well as MAPK activation in cultured vascular SMCs.

2. Pulmonary Hypertension

Reduced production of endogenous H_2S and CSE expression are believed to contribute to pulmonary hypertension and pulmonary vascular structure remodeling (282, 752). One animal model for pulmonary hypertension is induced by subjecting rats to an abdominal aorta-inferior vena cava shunt to create high pulmonary flow. Similar to what happened in essential hypertension animal model, plasma H_2S level and CSE mRNA level in pulmonary arteries and other lung tissues were downregulated 11 wk after shunting. Certainly, the altered endogenous H_2S metabolism in this case is secondary to increased pulmonary blood flow and hypertension (724). On the other hand, supplementation of NaHS to these animals seems to be beneficial as the high systolic pulmonary artery pressure (SPAP) was lowered by 20% and pulmonary remodeling was partially ameliorated. The percentage of muscular artery and the relative medial thickness of pulmonary arteries were decreased. These protective effects of H_2S were interpreted as the consequences of the inhibition of vasoactive peptides (endothelin-1, atrial natriuretic peptide, calcitonin gene-related peptide, and proadrenomedullin peptide), upregulation of HO-1/CO pathway (369), and the inhibition of pulmonary vascular inflammation (281, 282).

Another animal model for pulmonary hypertension in the context of H_2S study is the hypoxic pulmonary hypertension (HPH) (11, 451). HPH is closely linked to hypoxic pulmonary vasoconstriction. This unique hypoxic response in mammalian pulmonary blood vessels helps readjust lung blood supply according to the oxygen level. HPH is associated with many other hypoxic diseases, including chronic obstructive pulmonary diseases, mountain sickness, and sleep apnea syndrome, etc. The pathogenic mechanisms of HPH have not been clearly comprehended. Interaction of H_2S with NO and CO seems especially relevant for this disease situation. In a nutshell, H_2S may upregulate the HO/CO pathway and downregulate the NOS/NO pathway, so to limit the pathology of hypoxic pulmonary hypertension (695).

Intermittent exposure of rats to 10% oxygen for 6 h daily for 2–4 wk produced a chronic HPH with right ventricular hypertrophy (695). HPH was characterized with a mean pulmonary artery pressure (mPAP) of 25 mmHg or more and pulmonary capillary wedge pressure of 15 mmHg or less, both measured at rest by right heart catheterization. In this HPH model, CSE expression level and H_2S production in lung tissues were downregulated, and NaHS administration (intravenous) limited the development of HPH as the mPAP decreased by ~30%. Lowered level of GSSG and higher level of total antioxidant capacity in the lung tissues reflected NaHS-induced antioxidant protection (116, 695).

3. Renal Hypertension

In the two-kidney, one-clip (2K1C) rat model of renovascular hypertension, renin mRNA and protein expressions are upregulated and the activities of plasma renin and angiotensin II are elevated. These 2K1C-associated abnormalities as well as the renal hypertension were corrected by NaHS treatment (379). However, when plasma renin level is normal as in case of normal rats or one-kidney, one-clip (1K1C) rats, NaHS treatment had no effect on blood pressure or plasma renin activity. Therefore, the protective effect of NaHS in 2K1C rats appears to bear correlation with the basal activity of renin-angiotensin II system. This possibility was directly tested in primary cultured renin-rich kidney cells. As well known, the release of renin from the juxtaglomerular (JG) cells is regulated by intracellular cAMP. Forskolin stimulation of the cultured kidney cells increased renin activity in the medium and increased intracellular cAMP level. Addition of NaHS to the culture medium inhibited the effect of forskolin, providing the molecular mechanism for H_2S-induced inhibition of renin synthesis and release (379).

K. I/R and Myocardial Injury

The protection effect of H_2S against cardiac I/R damage has been shown in various animal species, including pigs (462), mice (90, 91, 160, 403), and rats (52, 206, 249). Different in vivo and in vitro heart I/R injury animal models as well as cellular models have been employed for studying the role of H_2S.

For example, one pig in vivo model was used with 60 min of mid-left anterior descending coronary artery occlusion and 120 min of reperfusion (462). In this study, cardiac myocyte apoptosis, together with the cleavage of caspase-3, was inhibited by H_2S. Interestingly, the researchers found that infusion of H_2S may offer better cardiac protection than a bolus administration of the gas in reducing myocardial necrosis after I/R injury.

One mouse in vivo model subjected the animals either to permanent ligation of the left coronary artery for 4 wk or to 60 min of left coronary artery occlusion. Thereafter, the hearts were reperfused for 4 wk (90).

One mouse model more closely relevant to heart attack created normothermic sudden cardiac arrest (CA), which was followed by cardiopulmonary resuscitation (CPR) with chest compression and mechanical ventilation. Seven minutes after the onset of CA, the mice received CPR (403).

One in vitro I/R injury model used isolated-perfused mouse hearts subjected to global I/R, and the protective role of Na_2S was studied and confirmed (403).

One cellular model of hypoxia-reoxygenation of isolated cardiomyocytes showed that NaHS protected the incubated cells from injury (250), which is mediated by ERK and Akt phosphorylation.

In all these cases, administration of H_2S or its donor limited infarct size, maintained ventricular function, and decreased circulating troponin I levels (91, 160, 462). Increased production of endogenous H_2S via the activity of CSE also protects the heart from I/R damage (160, 403, 571). On the other hand, blockade of endogenous H_2S production by PPG reduced the protective effect of ischemic preconditioning (53).

The cardiac protective effect of H_2S can be attributed to multiple mechanisms. H_2S improves cardiac blood supply via its vasorelaxant effect (773) as well as the enhanced NOS activity (403). Cell death during tissue hypoxia/ischemia and with consequent rapid reoxygenation/reperfusion is fundamental to the pathology of acute myocardial infarction (161). Both apoptosis and necrosis of cardiomyocytes can be inhibited by H_2S (160, 206, 462). Increased nuclear respiratory factor 1 and nuclear factor-E2-related factor (Nrf2) by H_2S during I/R injury has been reported, which helps attenuate apoptosis (90, 91, 284, 462). Activation of K_{ATP} channels by H_2S limited infarct size, which could be ascribed to increased blood supply to the ischemic tissues and to the anti-inflammatory actions of H_2S in the heart (160, 284). Blockage of mitochondrial K_{ATP} channels with 5-hydroxydeconoate abolished the anti-apoptotic effects of H_2S (571). Protein kinase C activation by H_2S is also linked to the opening of K_{ATP} channels in I/R injured cardiac tissues. The blockage of K_{ATP} channels with glibenclamide abolished H_2S-induced translocation of PKC isoform ε and isoform δ to the membrane fraction. Moreover, activated PKC can buffer intracellular calcium surges by stimulating the sodium/calcium exchanger and sarcoplasmic reticulum calcium ATPase mechanisms. Yong et al. (747) have reported that ischemic postconditioning offers protection for I/R injured rat hearts due to improved contractile function and increased PKC and PKG phosphorylation, which were abolished by PPG treatment (747). Anti-inflammatory effect is important for H_2S-offered cardiac protection as this gasotransmitter inhibits leukocyte transmigration and the expression and activities of inflammatory factors (160). H_2S treatment also reduced the activities of myocardial MOP, IL-1β levels, p38 MAPK, c-Jun NH_2-terminal kinase, and NF-κB (571). Also included in the antioxidant profile of H_2S are inhibited production of lipid peroxidation; increased expression of anti-oxidants HO-1 and thioredoxin 1; increased expression of heat shock protein 90, heat shock protein 70, Bcl-2, Bcl-xL, and COX-2; and inactivation of the pro-apoptogen Bad (91, 160). Increased phosphorylation of ERK1/2, Akt, and PI3K may also underlie the cardioprotective effect of H_2S (250). Finally, H_2S preserves mitochondrial structure and function during I/R injury by decreasing mitochondrial oxygen consumption and increasing complex I and complex II efficiency (160). Mitochondrial swelling was decreased and matrix density increased in mice receiving H_2S (160), and increased mitochondrial biogenesis (90).

A key strategic issue for application of H_2S-based therapy in myocardial I/R injury is the timing of H_2S delivery. Delivering H_2S at different stages of I/R injury may generate different prognosis of I/R injury.

1) Before ischemia (preconditioning): Calvert et al. applied Na_2S (100 μg/kg iv) to mice 24 h before myocardial ischemia (91). The cardiac protection was also achieved with H_2S infusion over the whole I/R period (462).

2) After ischemia but before reperfusion (postconditioning): In a mouse I/R model, Na_2S (100 μg/kg) was applied at the time of reperfusion (intracardiac) and then daily (intravenous) for the first 7 days after myocardial ischemia (90). This was the same protocol used in another mouse I/R model (160). Both treatments protected against the structural and functional deterioration of the left ventricle by attenuating oxidative stress and mitochondrial dysfunction.

Administration of Na_2S to the animals 1 min before CPR markedly improved survival rate at 24 h after CPR (15/15), prevented CA/CPR-induced oxidative stress, and ameliorated left ventricular and neurological dysfunction 24 h after CPR (403). Similar postconditioning protection was also reported in isolated rat hearts. Six episodes of a 10-s infusion of NaHS or 2-min continuous NaHS infusion before reperfusion improved cardiodynamics and reduced infarct size (747). It is interesting to take note that ischemic postconditioning (6 episodes of 10-s ischemia at the onset of reperfusion) also stimulated H_2S production from the isolated perfused rat hearts upon the start of reperfusion (747).

3) After reperfusion (Late conditioning): Administration of Na_2S did not protect the heart from I/R injury if the agent was given 10 min after CPR, i.e., after reperfusion (403).

These different schemes of H_2S intervention indicate that H_2S cannot reverse cardiac damage once the damage takes the shape and the window for H_2S conditioning is closed. Preconditioning and postconditioning with H_2S both protected the hearts from I/R injury. Both have their important clinical applications depending on the stage of I/R injury and the use of different clinical intervention procedures.

L. I/R and Liver Injury

Liver injury induced by I/R and its rescue by H_2S follow the same pattern as in myocardial I/R injury. Hepatic I/R increased CSE mRNA expression and the production of H_2S in rat livers (296). Although these compensatory outcomes were insufficient to protect the liver from I/R injury, without them it made hepatic I/R injury even worse as evidenced by the aggravated effect of PPG. Additional supplementation of H_2S donors (NaHS or IK1001) 5 min before reperfusion (274) significantly attenuated the severity of liver injury in both rat and mouse hepatic I/R models. The elevated levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were lowered by ~70% by IK1001 (274). These hepatic protective effects of H_2S are mediated by an array of molecular events, similar to the protective effects of H_2S on the I/R injured hearts, including antioxidative stress, anti-inflammation, antiapoptosis, etc. For example, an improved balance between GSH and GSSG was achieved by H_2S treatment together with an increased expression of thioredoxin-1 (274).

M. Neurodegenerative Diseases

As discussed in Section V, H_2S is endogenously produced in the brain from L-cysteine and modulates brain functions. The content of SAM, a CBS activator, is significantly decreased in the brains of AD patients (412), and blood homocysteine level increased in AD patients (119). With these abnormalities, CBS activity in the brain might be weakened and endogenous H_2S levels in AD brains lower than in age-matched healthy people. This hypothesis was later confirmed by Eto et al. (167). The levels of H_2S are noticeably lower in the brains of AD patients than that of age-matched normal individuals (167). There was no significant difference in the amounts of endogenous free cysteine or the expression levels of CBS between AD and control brains. However, the difference in homocysteine level between AD and control brains was not examined. Neuroinflammation has also been found to exacerbate hyperphosphorylated tau and amyloid-β (Aβ) generation by generating a plethora of inflammatory mediators and neurotoxic compounds in animal model of AD (221). These studies support the idea that reduced brain H_2S level may contribute to the pathological process of AD. With adequate H_2S in place, damages to PC12 cells by β amyloid would have been rescued (623). The inhibition of neuroinflammation by H_2S-releasing compounds is another indication for the correlation of H_2S level and AD (340). Decreased H_2S production would also jeopardize the antioxidant capacity of brain tissues since the elevated oxidative stress level is known in severe AD brains. On the other hand, supplementation of H_2S ameliorated the impaired cognitive impairment. In one rat study, bilateral intracerebroventricular injection of LPS decreased H_2S level and increased pro-inflammatory factors in the hippocampus. Treatment of these rats with *S*-propargyl-cysteine, a novel agent to increase H_2S production, resulted in improved Morris water maze performance and normalized H_2S level in rat hippocampus (212). Using H_2S as a therapeutic approach for AD has also received inspiration from the beneficiary effect of garlic extracts on animal models of AD. Garlic extracts contain organosulfur-containing compounds such as *S*-allylcysteine and di-allyl-disulfide (219). These compounds were reported to reduce cerebral inflammation and tau conformational changes in AD transgenic model. Amyloid-beta fibrillogenesis was also inhibited by these compounds (221). In this regard, the bioconversion of polysulfides in garlic to H_2S could link H_2S to the inhibition of AD development (43). To date, H_2S has not been directly applied to AD animal models.

Abnormalities in the cerebral microvasculature are relevant to the cause of dementia, including AD and vascular dementia (VD). Zhang et al. (767) employed a rat model of VD by occluding bilateral common carotid artery and vertebral artery for 5 min for three times with an interval of 5 min. One month after VD induction, the number of neurons in the hippocampus was decreased and neuronal apoptosis increased. Coincidently plasma H_2S level was gradually decreased. NaHS treatment (intraperitoneal) significantly reversed neuronal injury and improved functional performance of the animals through Morris water maze. NaHS also markedly increased Bcl-2 expression and decreased Bax expression. These neuronal protective effects of exogenous H_2S donor are suggestive of using H_2S as a therapeutic agent for VD. They are also indicative of a neuroprotec-

tive role of endogenous H_2S. In this regard, it was also found that hydroxylamine treatment (intraperitoneal) exaggerated the neuronal injury, exacerbated learning and memory, and reduced the ratio of Bcl-2/Bax in VD rats (767). Hydroxylamine is an inhibitor of CBS. It is expected that the inhibition of CBS decreases endogenous H_2S level, thus increasing neuronal apoptosis.

There is limited information about the role of H_2S in the initiation and development of PD. H_2S protects cultured PC12 and SH-SY5Y cells against various neurotoxins (6-OHDA, MPP+, and rotenone) via anti-oxidative, anti-neuroinflammatory, and anti-apoptotic mechanisms (246, 641, 744). In 6-OHDA- and rotenone-induced PD-like rats, H_2S levels in the substantia nigra and striatum are significantly lower. Supplementation of NaHS (30 and 100 μmol/kg) impeded the progression of movement dysfunction and preserved tyrosine hydroxylase-positive neurons in the substantia nigra (246). A mouse model of PD was also induced with a neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), featured with movement disorder and decreased tyrosine hydroxylase-containing neurons in the substantia nigra and striatum. The PD-like abnormality was prevented by inhalation of 40 ppm H_2S for 8 h/day for 7 days (303). H_2S treatment of PD animal inhibited the microglial activation in the substantia nigra, decreased proinflammatory factors such as TNF-α and NO in the striatum (246), and upregulated the expression of antioxidant proteins such as heme oxygenase-1 and glutamate-cysteine ligase (303). Overproduction of H_2S was also implicated in trisomy 21, the most common form of Down's syndrome.

The CBS gene is encoded on chromosome 21q22.3, which is a region associated with Down's syndrome. Therefore, it has been proposed that H_2S may be involved in the cognitive dysfunction associated with Down's syndrome. Loss of CBS activity also causes homocysteinuria, an autosomal recessive disease characterized, in part, by mental retardation. Finally, polymorphisms of CBS gene have been found to be significantly underrepresented in children with high IQ compared with those with average IQ, which hints that CBS activity could be involved in cognitive functioning (307). These observations, taken with the findings described previously, suggest that CBS and its product, H_2S, may regulate some aspects of synaptic activity and modify cognitive function.

IX. THERAPEUTIC APPLICATIONS OF H_2S

Great potentials of H_2S for dealing with different pathological situations inspire great efforts for exploring the best ways to deliver H_2S and to invent H_2S-based therapeutic strategies. In addition to releasing H_2S into the body fluid, other approaches have been attempted to increase endogenous H_2S level by providing substrates of H_2S-generating enzymes. Examples include L-cysteine and *N*-acetylcysteine. This section, however, focuses on the administration of exogenous H_2S.

A. H_2S-Donating Compounds

1. *H_2S donors*

A) H_2S FAST-DELIVERING COMPOUNDS. The simplest and most straightforward H_2S preparation is H_2S itself in either an aqueous form or a gaseous form. The bubbling of a physiological saline with pure H_2S gas yields a H_2S solution with known concentrations of H_2S within (772). The conventional preparation involves bubbling the saline with pure H_2S gas at 30°C for 40 min to obtain a H_2S-saturated solution (0.09 M) as step 1. Step 2 is to dilute this saturated solution further to the desired final concentration. Treatment of animals, tissues, or cells with this H_2S gas-bubbled solution has the advantage for the ready availability of pure H_2S gas and the bubbling apparatus as well as the simplicity for interpretation due to the lack of other nonsulfide elements in the solution. As H_2S is physically dissolved in the liquid for this preparation, the question is often asked as per the release of H_2S from the solution. This is a legitimate concern. H_2S can be released from the solution in a temperature- and concentration-dependent manner. At 37°C or below, the concentration of H_2S of the bath solution was relatively stable. When the H_2S-bubbled solution has the initial H_2S concentration below 1 mM, 15% decrease in H_2S concentration in the solution would occur within 30 min (772). Therefore, in most controlled experiments in a laboratory setting with room temperature around 22 or 37°C for cell culture experiments, the H_2S-bubbled solution can be reliably used as long as the solution will be refreshed frequently. One noticeable pitfall for the bubbled solution is the requirement of a well-ventilated environment that will quickly remove the discharged H_2S gas from the bubbling process, which sometimes becomes problematic.

Sodium hydrosulfide (NaHS) has been used as a H_2S donor more often than any other donors including H_2S-bubbled solution. The NaHS standard solution can be made by logarithmically diluting from freshly made 1 M NaHS stock solution. Several reasons are behind its wide utilization. One is its convenient preparation without pungent smell during the preparation process, simply adding NaHS compound to the liquid. Another one is based on the dissociation of NaHS into Na^+ and HS^- in solution. The latter can associate with H^+ to form H_2S. Given a physiological pH around 7.4 and temperature of 37°C, NaHS solution will yield about one-third of the undissociated H_2S and the other two-third remains as HS^- (39). Therefore, it is conventionally believed that the NaHS solution will give the researchers better control of the concentration of H_2S actually delivered than H_2S-bubbled solution. Remember, the same argument can also be made for H_2S-bubbled solution based on theoretical calculation. In the case of NaHS, one

has to be very careful in that the dissociation of NaHS is pH dependent and the generated H_2S can also be released from the solution in a temperature- and concentration-dependent manner. When the argument is made that in some experiments the final H_2S concentration was one-third of NaHS given, erroneous interpretation of the result will be generated if pH was not adjusted to ~7.4 for both the NaHS solution per se or the medium in which NaHS was added. Unfortunately, many published papers do not mention this important pH adjustment when NaHS was used. Finally, NaHS and H_2S-gassed solution exhibit some different biological effects at certain concentration ranges. Therefore, some effects of NaHS cannot be fully explained by H_2S. Altered Na^+ concentration or ionic strength as well as the redox potential may be associated with NaSH solution, especially at high end of the concentrations used.

Sodium sulfide (Na_2S), also under the name of IK-1001, has been used as a H_2S donor in a number of studies (265, 499, 755). This salt is soluble in water, but not in ethanol as NaHS is. This differential solubility can be used to separate NaHS from Na_2S and sodium thiosulfate. Once in solution, Na_2S dissociates and generates H_2S. In addition to having one more choice in using H_2S donors, it is not clear whether Na_2S is superior to other donors (755). It has not been determined how much H_2S would be released at a pH of ~7.4.

Lawesson's reagent [2,4-bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane 2,4-disulfide], originally used in organic synthesis as a thiation agent (338), has been applied in biological studies as a H_2S donor in few reports. Lawesson's reagent can be obtained commercially. Chlorine bleach is often required to eliminate the foul-smelling residues after its preparation. Lawesson's reagent (0.1–3 μmol/kg) has been used to assess the effects of H_2S on aspirin- and fMLP-induced leukocyte adherence (755), on neutrophil migration (137), on carrageenan-induced knee joint synovitis (158), and on ulcer healing (670), for example. However, it is not clear how much H_2S is actually generated by Lawesson's reagent under in vivo conditions in these studies. It has been reported that NaHS, Na_2S, and Lawesson's reagent have different potencies for the same cellular event (755), which indicates that these donors release different amounts of H_2S and/or different H_2S donors generate different cellular effects not related to the released H_2S.

Another H_2S donor reported is 4-hydroxythiobenzamide (4-HTB) (105, 670). Due to the limited studies, it is difficult to assess its advantages or disadvantages over other H_2S donors.

An intrinsic challenge for applying H_2S-fast releasing compounds is the difficulty in achieving the desired final concentration of H_2S without generating waveform-like H_2S concentration fluctuations. In cell culture or tissue/organ culture studies, NaHS or Na_2S solutions can be added to the culture dishes or chambers as a concentrated stock solution or by completely changing the solution to the one already containing dilute NaHS/Na_2S. In either case, the final concentration of H_2S in the solution cannot be maintained at constant level over a relatively long period. In the whole animal study, the issue will be more challenging as very often, a bolus injection of the animals with NaHS/Na_2S stock solution is conducted. At the moment of the injection, the injection site or the circulation will see a great surge of H_2S level, but thereafter, a much lower and declining level of H_2S is encountered and declining. This may explain in some studies the biphasic or multiphasic reactions of the biological systems to NaHS/Na_2S treatment.

B) H_2S SLOW-RELEASING COMPOUNDS. The fast delivery of H_2S by H_2S-bubbled gas or NaHS challenges the situations where a long-term sustained supply of H_2S is required and where a fast H_2S overshoot surge followed by rapid decline is undesirable. To answer this call, H_2S-releasing compounds have been developed in the last several years. The examples of these slow-releasing compounds are GYY4137 (362) and *S*-diclofenac (361, 561).

The rate of hydrolysis of H_2S donors has specific applications on different organs and for different abnormalities. To achieve a sustained therapeutic outcome is one of the reasons why the H_2S slow-releasing compounds are welcomed. Therapeutic effectiveness also depends on the rate of H_2S delivery. A good example could be the dilemma of anti- or pro-inflammatory role of H_2S. The slow releasing of H_2S from GYY4137 inhibited LPS-induced release of pro-inflammatory mediators and increased the synthesis of the anti-inflammatory chemokine IL-10. The rapid action of NaHS at high concentrations, in contrast, increased the synthesis of proinflammatory factors (706). Another example is with *S*-diclofenac. While NaHS is pro-angiogenetic (400, 467), H_2S slow-releasing *S*-diclofenac inhibits angiogenesis and endothelial cell proliferation (267).

GYY4137 [morpholin-4-ium 4 methoxyphenyl(morpholino)phosphinodithioate] is a novel water-soluble compound that releases H_2S slowly and steadily, either in aqueous solution or administered to the animal (intraperitoneal or intravenous) (362). The slow-releasing property of GYY4137 results in slow vasorelaxation without affecting heart rate or myocardial contraction of rats. Also due to this slow-releasing profile, GYY4137 inhibited the development of hypertension ina N^G-nitro-L-arginine methyl ester (L-NAME)-evoked rat hypertension model or in SHR rats, after 14 days of administration (362).

GYY4137 was applied to cell culture medium every 3 days to maintain the H_2S level (750). This is in sharp contrast to the application of NaHS or Na_2S or H_2S-bubbled solution to cell culture study, in which these fast-delivery H_2S do-

nors should be given at least twice daily. A bolus injection of GYY4137 to mice (intraperitoneal) quickly increased H_2S concentrations in the liver and heart for the enduring 20 min, which is even longer in the kidney (750). It should be noticed that when using the H_2S slow-releasing compounds chronic accumulation of H_2S in the targeted cells or tissues could be generated, which may be detrimental under certain conditions (750).

There is another significant difference between H_2S fast-delivery compounds and H_2S slow-releasing compounds in addition to the rate of hydrolysis: that is the membrane permeability. H_2S fast-delivery compounds would easily pass the cell membrane and blood-brain barrier, and it is not sure for H_2S slow-releasing compounds in this aspect. Should the slow-releasing compounds fail or be too slow to pass the blood-brain barrier, the released H_2S outside the brain would also be metabolized or scavenged before it reaches the brain. This may explain why the H_2S concentration in the brain did not change after GYY4317 injection (750). Is this a bad thing? It really depends on the situation. When organ-selective administration of H_2S is required, this property would be a plus, but when the brain is the target for H_2S treatment, it becomes a minus.

2. Hybrid Molecules Between H_2S and the Compounds With Known Structures

H_2S-releasing hybrid compounds are created by combining a H_2S-releasing moiety with another parent compound with known molecular structure and biological functions. The purpose to create such a new compound is to enhance the functionality and safety of both compositing compounds and reduce potential side effects of each moiety. To date, this purpose appears to be partially achieved from animal studies. The challenges for these H_2S hybrids molecules include the analysis of the pro and con for their chronic usage, H_2S releasing kinetics, metabolic dynamics in vivo, and accessibility to different organs and tissues. And, of course, the final test of their usefulness would be the clinical trials.

In most cases, the H_2S-releasing hybrids are made by grafting onto existing compounds the moiety 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADTOH), the main metabolite of ADT (209). Some examples of these newly invented H_2S moieties are reviewed below.

A) ACS14 (S-ASPIRIN). Hybrid nature: between ADTOH and aspirin. Rationale: while protecting the cardiovascular system, aspirin has the tendency to cause gastric damage. Advantage: ACS14 inhibited thromboxane synthesis, similar to aspirin, but caused much weaker gastric reactions than aspirin did (594). This could be due to the antioxidant effect of H_2S, released from the hybrid. Another related compound with S-aspirin is ACS21, S-salicylic acid. Both ACS14 and ACS21, but not aspirin and salicylic acid, limited the development of metabolic syndrome in rats induced by GSH depletion (including hypertension, endothelial dysfunction, and insulin resistance), and protected the heart from I/R damage. And no gastric lesion was found with the application of ACS14 or ACS21 (527, 528). Oral administration of ACS14 for 7 days or a single intraperitoneal injection to rats significantly increased GSH levels in heart, aorta, and plasma (209, 584). This effect on thiol metabolism was also achieved with ADTOH, but was not with aspirin.

B) ACS6 (S-SILDENAFIL). Hybrid nature: Between ADTOH and sildenafil. Rationale: Sildenafil is an effective and selective inhibitor of type 5 phosphodiesterase (PDE5), of which the location in corpus cavernosum and prostate is especially related to abnormalities in male reproductive system and urinary system. With increased oxidative stress level and NOX expression, such as in diabetes, the efficiency of sildenafil in treating erectile dysfunction is compromised. Advantage: Both ACS6 and sildenafil relaxed cavernosal smooth muscle, but ACS6 is more potent in inhibiting the formation of superoxide and expression of p47(phox) and PDE5 than sildenafil (560). This hybrid will take the advantage of sildenafil-maintained cGMP activity and H_2S-activated K_{ATP} channels and suppressed oxidative stress in penile tissues, leading to an enhanced potency in relaxing penile cavernosal muscles. This hybrid may also find its application to those patients with erectile dysfunction, who do not respond to sildenafil treatment. Furthermore, NaHS and ACS6 both inhibited superoxide formation in cultured porcine pulmonary arterial endothelial cells (PAECs), but ACS6 is about at least 10 times more potent than NaHS (422). Both 100 nM NaHS and 1 nM ACS6 completely inhibited gp91(phox) expression induced by TNF-α. The effects of NaHS and ACS6 may not be mediated by the same signaling pathways, since inhibition of PKA blocked the effect of NaHS but the blockade of both PKA and PKG abolished the effect of ACS6, confirming the involvement of both H_2S and NO-cGMP signals with ACS6. Since sildenafil has been tried in clinical settings for the treatment of pulmonary hypertension, the efficacy and potency of ACS6 for the same would be interesting to test.

C) ACS84 AND OTHER H_2S-RELEASING L-DOPA DERIVATIVES, *S*-DOPAS. In this category, we have ACS83, ACS84, ACS85, and ACDS86 (341). Only ACS84 is discussed here in details as an example.

Hybrid nature: Between H_2S-donating dithiolthione or allyldisulfide and L-DOPA methyl ester. Rationale: While extensively being used for treatment of PD, L-DOPA (Levodopa) does not have the capacity to prevent or decrease the apoptosis of substantia nigra dopaminergic neurons. On the other hand, H_2S protects neurons from apoptosis via its anti-oxidant and anti-inflammatory effects (see sect. VIII). Advantage: ACS84 injection (intravenous) in rats delivered

more dopamine to the brain than L-DOPA did. With cultured human microglia, astrocytes, SH-SY5Y neuroblastoma cells, and humanTHP-1 U373 cell lines, ACS84 increased intracellular H_2S levels. ACS 84 reached the brain and was substantially metabolized as early as 1 h after administration to rats intravenously, causing more than 2-fold increase in brain dopamine and 1.4-fold increase in GSH (341). In fact, ACS84 has a better permeability to blood-brain barrier than L-DOPA. Additional benefits of ACS84 over L-DOPA are the decreased release of pro-inflammatory factors and reduced cell death (341).

D) ACS67 (S-LATANOPROST). Hybrid nature: Between a H_2S-donating moiety dithiolethione (ACS1) and latanoprost. Rationale: Latanoprost is a synthetic derivative of the natural prostaglandin $F_{2\alpha}$. As an agent to lower intraocular pressure (IOP) in glaucoma management, latanoprost does not protect retina from ischemia damage, and its tolerance by patients is low. Advantage: Among retinal damages after ischemia are altered ERG, reduced retinal localization of specific antigens, and decreased optic nerve axonal proteins. Intravitreal injection of ACS67 immediately after ischemia blunted most of these abnormalities. ACS67, but not of latanoprost, attenuated the death of cultured retinal ganglion cells (RGCs) by increasing GSH levels and decreasing H_2O_2 toxicity (461). This observation from cultured cells was supported by the whole animal study in glaucomatous (carbomer model) pigmented rabbits. In these animals, ACS67 achieved a greater anti-IOP effect than latanoprost. Moreover, the rabbits tolerated ACS67 well over a 5-day treatment regimen with daily intraocular administration (480). The argument is made that the additional neuronal protective effect of ACS67 compared with latanoprost is due to the release of H_2S from the hybrid. However, it should be noted that latanoprost itself can increase the viability of RGCs and stimulate retinal neurite outgrowth (775).

E) ACS15, S-DICLOFENAC OR CODED AS ATB337. Hybrid nature: Between ADTOH and diclofenac. Rationale: Diclofenac is one of the NSAIDs. While inhibiting inflammation and eliciting analgesia, NSAIDs also may cause GI toxicity among other side effects. This undesired side effect of NSAIDs has been shown to be minimized by H_2S donors in the rat stomach (136). Furthermore, the application of NSAIDs would decrease endogenous H_2S synthesis (180). Advantage: ACS15 is more potent than diclofenac for anti-inflammatory activity in animal studies. At the same time, the side effects of diclofenace, such as neutrophil infiltration, leukocyte adherence, and GI lesion (361, 561), as well as pancreatitis-related lung injury (48) were significantly reduced with ACS15.

With the same principle and expectations, other H_2S-NSAIDs hybrids, or S-NSAIDs, have been made with the codes like ATB-343 (derivative of indomethacin), ATB-345, ATB-346 (derivative of naproxen), and ATB-429 (derivative of mesalamine). All these S-NSAIDs, they are metabolized by carboxylesterases in the body to slowly generate H_2S. For details about the structures and their applications, readers are referred to some excellent reviews on this topic (89, 670). **FIGURE 6** depicts the chemical structures of some representative H_2S donors.

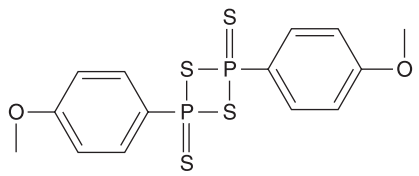
3. Inhalation of H_2S Gas

Inhalation of H_2S gas has been attempted in animal studies to induce suspended animation, to reduce the risk of hemorrhage, and to enhance effects of deliberate hypothermia during anesthesia and mechanical ventilation after brain trauma or circulatory arrest. Whether inhalation of H_2S gas can be a useful and important therapeutic strategy for humans has not been seriously tested, but a few considerations are worth discussing as a way of forward thinking. The safety for inhaling H_2S gas is always a key issue as inhaled H_2S has been identified as an industrial and environmental risk (for more details, please see sect. II). As long as the dosage of H_2S gas is controlled at a safe level, this can be handled safely.

Inhaled H_2S enters circulation through the respiratory system and will be dissociated in part into hydrosulfide ions. The free H_2S remaining in the blood interacts with metalloproteins, disulfide-containing proteins, and thio-*S*-methyltransferase and forms methylsulfides (39, 240). The hydrosulfide ions then bind to heme compounds and are metabolized by oxidation to sulfate. The interaction between the hydrosulfide ions and methamoglobin, which forms sulfmethemoglobin, is a detoxification pathway (575).

The vulnerability of nasal epithelium is another concern for H_2S-induced pathology. Injury to the nasal respiratory mucosa occurs in animals with ongoing H_2S exposure. On the other hand, the regeneration of respiratory epithelium can adapt these cells to become resistant to the subsequent injury. Roberts et al. (520) exposed Sprague-Dawley rats nose-only to 200 ppm H_2S for 3 h/day for as long as 5 consecutive days. This nasal exposure indeed altered the expression profiles of a number of genes in epithelial cells, including those involved in cell cycle regulation, protein kinase regulation, and cytoskeletal organization and biogenesis. Keep in mind that 200 ppm is already toxic and the exposure duration in this study may also be beyond the human tolerance. Nevertheless, there was no significant change in cytochrome oxidase gene expression or bioenergetics in these exposed cells.

When and where the subject should inhale H_2S gas is also an important consideration. During anesthesia and mechanical ventilation, supplement of H_2S gas may help lower core temperature or reduce energy consumption. Baumgart et al. (36) found that awake mice inhaling H_2S exhibited reduced energy expenditure, hypothermia, and bradycardia



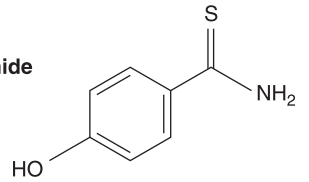
Lawesson's reagent

NaHS

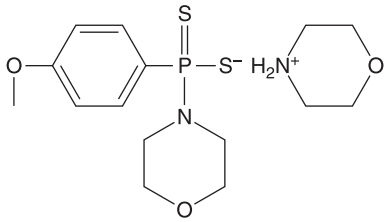
Sodium hydrogen sulphide

Na_2S

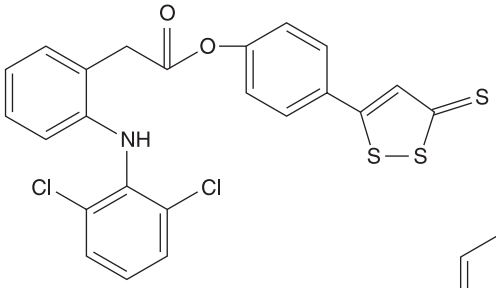
Sodium sulphide



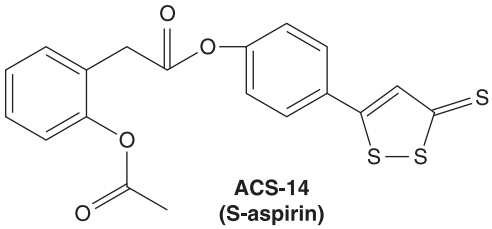
4-hydroxythiobenzamide



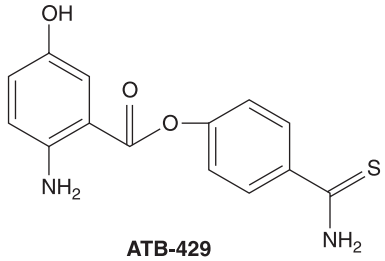
GYY4137



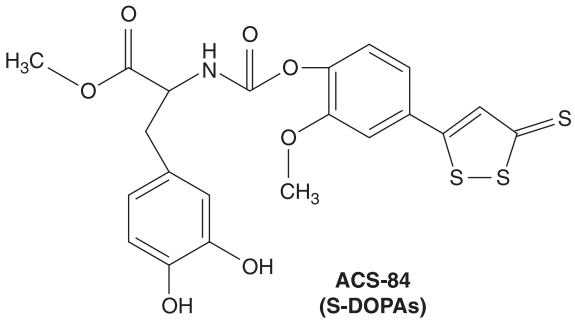
S-diclofenac
(ACS-15, ATB-337)



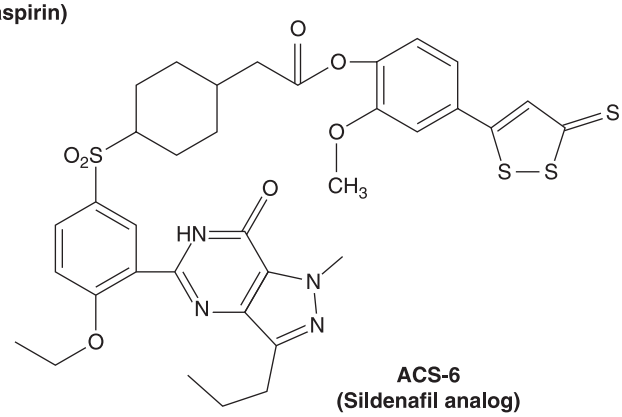
ACS-14
(S-aspirin)



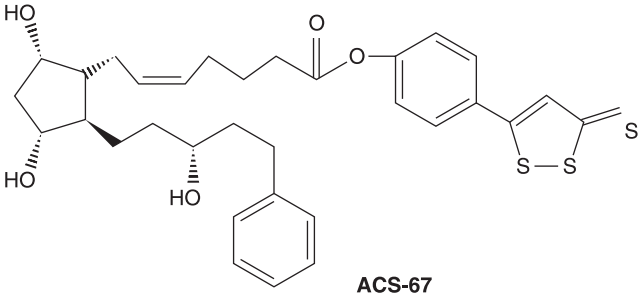
ATB-429
(Mesalamine analog)



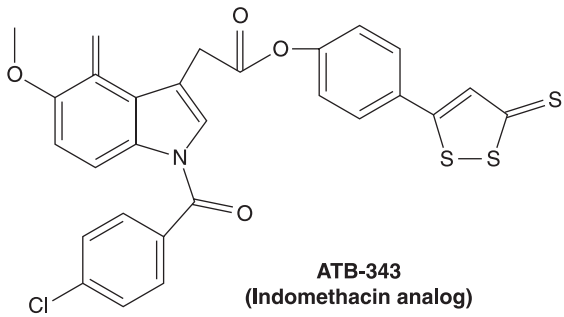
ACS-84
(S-DOPAs)



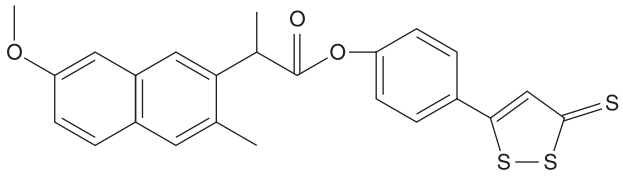
ACS-6
(Sildenafil analog)



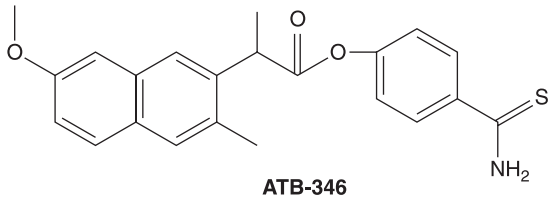
ACS-67
(S-latanoprost)



ATB-343
(Indomethacin analog)



ATB-345
(naproxen analog)



ATB-346
(naproxen analog)

FIGURE 6. Chemical formulas and structures of representative H_2S donors.

in spite of unchanged systolic heart function. The hypothermia may not be caused by H_2S gas in human, but decreased energy demand and preserved mitochondrial integrity will certainly reduce tissue or organ damage during surgery or organ transplantation. Inhalation of H_2S gas may also stand in the frontline of emergency aid.

Inhaled H_2S gas may be used to reversibly depreciate metabolic activity. In a controlled lethal hemorrhage in rats, inhalation of H_2S gas or intravenous infusion of NaHS increased the survival rate of the animals more or less to the same degree (413). The severe hemorrhage case might be extrapolated to other accidents or natural disasters where inhalation of H_2S can be performed on site or in ambulance by trained professionals, with or without oxygen gas inhalation together.

Many respiratory diseases may be specifically handled with H_2S inhalation. In this way, the inhaled H_2S gas would first come in contact with the lungs and respiratory tract. The metabolism of inhaled H_2S in the respiratory system is rapid. Fifteen minutes after inhalation of 400 ppm H_2S, the sulfide level in the lungs returned to the baseline level already. The olfactory epithelium and nasal epitheliual cells reacted with the inhaled H_2S (30 ppm or above) with decreased cytochrome oxidase activity, but the sulfide level in the olfactory epithelial cells was not increased until inhaled H_2S reached 400 ppm. Acute H_2S inhalation did not change the sulfide level in hindbrain and nasal respiratory epithelium. Quite interestingly, cytochrome oxidase activity after inhaling 10 ppm H_2S and sulfide concentration after inhaling 200 ppm H_2S were increased in liver (153). A similar study showed reversible lesions in the respiratory and olfactory mucosa of the rats after acute exposure to $>$80 ppm H_2S (67).

B. Potential Dietary Supplementation of H_2S

Nutritional supplements have found wide application and appreciation in recent years for promoting health, preventing disease, and treating disorders. In our daily life, many dietary elements directly and indirectly provide H_2S to our body so that the advantage of this gasotransmitter can be realized, in many cases, without conscious thought. Some of these nutritional sources for H_2S supplementation are reviewed below.

1. Garlic: Polysulfides and S-ally-L-Cysteine

Garlic (*Allium sativum*) offers assistance in lowering blood pressure and protecting the heart (43), fighting with atherosclerosis, lowering blood sugar and cholesterol levels (416), and preventing platelet aggregation. Garlic has also been found to be effective against bacterial, viral, fungal, and parasitic infections, in addition to enhancing the immune system and having antitumoral and antioxidant features (126). In a 1997 study involving more than 200 German men and women, half of the participants took 300 mg or more of standardized garlic powder in tablet form daily for 2 yr. The aortas of those 70-yr-old garlic-eating people were just as elastic as those of 55-yr-old non-garlic-eating people. The mechanism underlining the improved aortic was not clear, but researchers observed that "the only side effect of eating garlic is the odor" (65). Well, it may turn out that it is this "odor" that actually does the job (686).

For a long time, the pungent aroma of garlic has been noticed and attributed to volatile sulfur-containing flavor compounds, but how much credit of the health benefits of garlic should be given to these volatile compounds and what are their final sulfur products have not been clear. Among these sulfur-containing compounds are polysulfides, such as diallyl disulfide (DADS) and diallyl trisulfide (DATS); alk(en)yl-thiosulfinates formed by the action of allinase; *S*-allylcysteine (SAC); *S*-alk(en)yl-L-cysteine sulfoxides; and allicin (allyl-2-propenethiosulfinate), formed from the precursor allicin (*S*-2-propenyl-L-cysteine sulfoxide). The latter is the predominant garlic thiosulfinate and a potent platelet inhibitor (69). Many of these compounds are permeable to cell membranes to impact on cellular events (405).

Organic polysulfides can interact with either exofacial membrane protein thiols or intracellular thiols, such as GSH, to form H_2S. When fresh garlic extracts interacted with red blood cells, H_2S formation was ignited, and the gas was released as measured in real time by a novel polarographic H_2S sensor. In fact, garlic and garlic-derived polysulfide can induce H_2S production in biological membranes or intracellular milieu as long as the reduced thiols exist (43). A couple of nonenzymatic mechanisms have been proposed to explain the chemical conversion of garlic-derived organic polysulfides to H_2S. Benavides et al. (43) showed that under physiologically relevant oxygen levels rat aortic tissues also metabolized garlic-derived organic polysulfides to liberate H_2S. One conversion mechanism involves a sequence of reactions, from the formation of *S*-allyl-glutathione and allyl perthiol to allyl-GSSG and to H_2S (418, 597). Both allyl-GSSG and allyl perthiol can undergo nucleophilic substitution at the α-carbon to produce H_2S. Another mechanism, a lesser likelihood, is the direct thiol/disulfide exchange during the interaction of polysulfides and GSH.

In an acute myocardial infarction (AMI) rat model, pretreatment of the animals with *S*-allylcysteine (SAC) (50 $mg \cdot kg^{-1} \cdot day^{-1}$) for 7 days before AMI induction protected partially the heart from ischemia damage and saved the animals from mortality by ~11%. These effects were antagonized by PPG inhibition of CSE (115). Increased plasma H_2S level was detected in these rats after SAC treatment. The authors correlated these observations and hy-

pothesized that SAC may serve as the substrate for CSE and upregulate the CSE production, leading to increased plasma H_2S level and cardioprotection. However, whether SAC can be directly converted to H_2S was not attended (115).

Garlic-formed H_2S has been shown to significantly relax the isolated rat artery tissues (43). The lowering of systolic blood pressure by consumption of garlic has been known in human and animals (510). Direct administration of allicin (8 mg/kg) to rats also led to lowered systolic blood pressure and triglycerides (159). Garlic extracts, fresh or boiled, can reduce β-amyloid fibril formation and defibrillate them in AD transgenic model (221), which may be closely related to the anti-AD effect of garlic-derived H_2S. The inhibition of angiotensin converting enzyme (554), prevention of vascular remodeling (657), and suppression of L-NAME-induced hypertension (476) are other examples of the therapeutic potential of garlic compounds. As such, H_2S production from the garlic-derived organic polysulfides may underlie the long-term beneficial effects of dietary consumption of garlic on cardiovascular and neuronal systems. It has been calculated that two cloves per meal will probably release sufficient H_2S for maintaining the balanced blood vessel constriction. The older we are however, the lower H_2S production in our bodies, and the more prone we are to cardiovascular problems. Supplementary H_2S has the potential to provide innumerable benefits to aging populations.

2. Durian

As the "king of fruits" in many Southeast Asian countries, durian (*Durio zibethinus Murray*) is a notably flavorful, or pungent, fruit, depending on one's tastes. Not only served fresh, durian is also an element in many Southeast Asian cookies, ice creams, or candies. However, the penetrating odor of durian is so offensive that its possession in many public establishments in Thailand, Philippines, and Singapore, such as airports, hotels, and public transportation, is unlawful. Durian's increasing popularity even in distant markets as the United States and European Union ensues the intensification of research to inform and familiarize consumers on the characteristics of the fruit. The ripe durian has one distinct offensive smell, in addition to one strong and onionlike aromas and one with delicate and fruity, due to H_2S and diethyldisulfide.

Novel findings on its bioactive composition and health benefits (100) point to its potential use in disease preventive diets (350). Durian is rich in sulfur compounds (664, 665). Baldry et al. (26) identified a total of 26 volatiles in the distillate of durian fruits from Singapore and Malaysia, of which 7 are sulfur compounds. Later study detected eight sulfur compounds, mostly dialkyl polysulfides, in the headspace fraction of durian from Thailand, in which diethyl disulfide and diethyl trisulfide were predominant (414). The levels of the main sulfur compounds, except ethanethiol, increased with maturity. Wong and Tie (713) identified 63 volatiles in durian from Malaysia, of which 16 of which were sulfur compounds.

3. Thousand-Year Egg

Also known as pidan, thousand-year egg is a Chinese delicacy made from duck, quail, or chicken eggs preserved in a mixture of clay, ash, lime, salt, and rice straw. After the process is completed for several months, the yolk becomes a dark soft, greenish substance that exudes an odor similar to ammonia and sulfur, while the white becomes a dark brownish transparent jelly with little flavor. It can be eaten without extra preparation as a dim sum or side dish and is popularly used to garnish rice congee or porridge.

Consumption of thousand-year egg has been proven to supply the body with H_2S. Chau et al. (104) collected the thousand-year eggs from Hong Kong supermarket and used a gas chromatography/mass spectrometry (GC/MS) to analyze the egg's volatile compounds. H_2S and acetaldehyde were identified in thousand-year eggs and in ordinary cooked duck eggs. This should not be a surprise, since the sulfur amino acids are common in high protein foods such as eggs. During the fermentation process, H_2S can be generated from these sources of sulfur. Red meat, cheese, milk, fish, soybeans, preserved bean curd (stinky tofu), or nuts are all high-protein foods. Another source of exogenous sulfur is inorganic sulfate used as preservatives in processed foods. Consumption of these foods, such as commercial breads, beers, sausages, and dried fruit (185), ignites fermentation in the colon to generate H_2S by colonic bacteria or by sulfate-reducing bacteria (207).

4. Soured Herring

Yet another dish, this time from Sweden, turning fresh cuisine into rotten food to increase H_2S production is Surströmming, or "soured herring" (259, 649). This salted and fermented Baltic herring is a Swedish delicacy, mostly produced along the northern coast of the country but rapidly gaining popularity outside its borders. The fermentation process for soured herring can take up to a year, by the end of which the fermentation cans are dramatically swollen due to the accumulated gases inside. One should carefully open the cans under the water and outdoors to prevent the explosion and to reduce the impact of the stench, largely from H_2S and sour acetic acid. Then, this dish can become a great treat for its intense taste, not smell.

4. Broccoli and Sulforaphane

Broccoli as well as other cruciferous vegetables tends to release strong smell of H_2S when cooked or became rotten. Previous studies have reported the health beneficial effects of broccoli on the prevention and treatment of hypertension and atherosclerotic changes in the SHR stroke-prone rats

(715). These effects of broccoli are largely ascribed to the action of sulforaphane, which is a sulfur-containing compound that exhibits anticancer properties, and young sprouts of broccoli are particularly rich in sulforaphane. In a recent study by Pei et al. (477), the release of H_2S from sulforaphane was unmasked under different experimental conditions, such as the addition of sulforaphane to the cell culture medium or mouse liver homogenates or administration of sulforaphane to the living mice. While sulforaphane speeded the death of PC-3 cells (a human prostate cancer cell line) in a dose-dependent manner, scavenging of free H_2S with methemoglobin or oxidized glutathione reversed sulforaphane-reduced cell viability. The anti-prostate cancer effect of sulforaphane was also mimicked by NaHS treatment as PC-3 cell migration was inhibited by NaHS. The activation of p38 MAPK and c-Jun NH_2-terminal kinase (JNK) appears to be the common signaling linkage for the effect of sulforaphane and NaHS in PC-3 cells. Suppression of both p38 MAPK and JNK reversed NaHS- or sulforaphane-reduced viability of PC-3 cells.

Stinky but healthy, H_2S uptake via diet appears to be a good trade-off. Consumption of garlic, stinky tofu, thousand-year egg, or durian all increases the production of H_2S. The efficacy and efficiency of the nutritional supplementation of H_2S to deal with various health issues linked to H_2S deficiency have not been systematically and purposely examined. Our understanding in this regard largely remains at the anecdotal level and limited to cellular or animal studies. The intervention studies which directly link the dietary supplementation of H_2S-containing functional foods or fruits to pathological situations will be welcomed in the future.

X. CELLULAR AND MOLECULAR MECHANISMS FOR H_2S EFFECTS

A. K_{ATP} Channels and Other Ion Channels

The activation of K_{ATP} channels by H_2S is among the first identified molecular mechanisms for the cellular effects of H_2S. More details about this molecular mechanism are discussed in the next section.

B. cAMP and PKA Pathway

The activation of G_s protein-coupled membrane receptors stimulates membrane-attached adenylyl cyclase (AC), which catalyzes the production of cAMP from ATP. Protein kinase A (PKA) will be activated by cAMP and move on to phoshorylate a number of signaling or acceptor proteins. The end result is the altered cellular function. The decomposition of cAMP to AMP is mediated by the enzyme phosphodiesterase (PDE).

Among the molecular targets of cAMP/PKA pathway are ryanodine receptor on endoplasmic reticulum and the Ca^{2+} release-activated Ca^{2+} (CRAC) channels on plasma membrane, of which the activation leads to increase in intracellular calcium level. In the CNS, this can be achieved by PKA-induced phosphorylated NMDA receptors. The potentiation of both the early and late phases of LTP then ensued (518, 640). NaHS induced LTP in rat hippocampal slices by enhancing the NMDA-induced inward current and by increasing the sensitivity of NMDA receptor to its ligand in a cAMP-dependent manner (1). The production of cAMP in primary cultured cerebral cortex and cerebellum neurons and glial cells was increased by NaHS (1–100 μM). With NMDA receptors heterologously expressed in *Xenopus* oocytes, it was shown that NaHS (10–30 μM) significantly increased intracellular cAMP and decreased the onset of NMDA-induced membrane currents (306). H_2S-induced increase in $[Ca^{2+}]_i$ in microglia was attenuated by inhibition of PKA (344). Together, these observations support the notation that the effect of H_2S and the activation of cAMP pathway, NMDA receptors, and LTP are causatively linked. Increased cAMP level in transformed astroglia by NaHS was also reported (427). Further evidence for the involvement of cAMP pathway in neuronal effect of H_2S came from the studies on frog neuromuscular junction where NaHS (100 μM) induced neurotransmitter release. The effect of NaHS diminished in the presence of the membrane-permeable cAMP analog pCPT-cAMP (100 μM) (569), which suggests that cAMP and NaHS may share the same signaling pathway(s). However, inhibition of AC by MDL-12330A did not alter the effect of NaHS on neurotransmitter release. This can be explained if H_2S targets the downstream signaling molecules of AC. In the same study, it was shown that NaHS effect on end-plate currents was not mediated by cGMP/PKG pathway, since the presence of a membrane-permeable cGMP analog pCPT-cGMP or an inhibitor of guanylate cyclase, ODQ, did not alter the effect of NaHS.

Another signaling pathway activated by cAMP/PKA in the CNS is the PI3K/Akt/p70 ribosomal S6 kinase (p70S6K). NaHS has been shown to increase cAMP concentration and expression of PI3K, Akt, and p70S6K in isolated rat hippocampal neurons. The inhibition of Akt and p70S6K counteracted the anti-apoptotic effect of NaHS (555).

H_2S may also decrease cAMP production by inhibiting AC. This appears to be the case with renovascular hypertension development. The release of renin from the juxtaglomerular cells is a cAMP-dependent process. Increased renin release is causative for renovascular hypertension such as in the 2K1C rat model. These hypertensive rats showed a repressed development of renovascular hypertension after NaHS treatment (0.56–5.6 mg/kg). NaHS also decreased plasma renin activity and angiotensin II levels, inhibited the upregulation of renin expression, and lowered the high level of cAMP in the clipped kidneys of 2K1C rats. However, it is not clear whether the lowered cAMP level is secondary to

the lowered blood pressure after NaHS treatment. Direct measurement of cAMP level showed that NaHS (100 μM) decreased forskolin-increased cAMP production and renin activity in primary cultures of juxtaglomerular cells (379).

In muscle cells, the activation of cAMP/PKA pathway inactivates myosin light-chain kinase via its phosphorylation. Thus myosin light chain will not be activated to trigger muscle constriction. Therefore, the functional consequence of the activation of cAMP/PKA pathway in vascular tissues is vasorelaxation. There is no evidence to date that the vasorelaxant effect of H_2S involves cAMP/PKA pathway. The blockade of cAMP pathway would not alter the vasorelaxant effect of H_2S (311, 772). In cultured porcine pulmonary arterial endothelial cells, NaHS inhibited superoxide formation with IC_{50} of ~10 nM. The expression of gp91(phox) induced by TNF-α was also inhibited by NaHS. These effects of NaHS were likely mediated by cAMP/PKA pathway, since the inhibition of PKA, but not other pathways including PKG, blocked the effect of NaHS (422).

A role of downregulated cAMP in the vasoconstrictive effect of H_2S has been suspected. In one study, phenylephrine-precontracted rat aortic rings were relaxed by stimulation of α-adrenoceptors or by forskolin. The addition of NaHS at concentrations of 10–100 μM further constricted the vascular tissues. NaHS (5–100 μM) also significantly reversed forskolin-induced cAMP accumulation in a cell line of rat aortic vascular smooth muscle cells. The decreased cAMP in vascular smooth muscle cells (372) and in juxtaglomerular cells (379) was observed in the same lab. The very research team also reported a similar suppression of cAMP level by H_2S in cardiomyocytes (747). Whether NaHS directly inhibited AC or stimulated PDE in these studies was not tested. Confirmation of this phenomenon from other research labs is also required. Differential effects of H_2S on cAMP/PKA pathway have been related to the expression of different isoforms of AC and/or PDE in different types of cells. However, this would not explain why in the same vascular smooth muscle cells H_2S would decrease cAMP level in one case but have no effect in other cases. It should also be aware that H_2S may nonspecifically inhibit PDEs (76). The consequence of this effect was shown in a recent study in which NaHS at nanomolar concentrations lowered the levels of 5′-AMP, the hydrolytic products of cAMP, in smooth muscle cells, indicating that cAMP levels are increased. The integration of the opposite effects of H_2S on AC and PDE would affect the net cAMP level, which remains not clear.

C. cGMP and PKG Pathway

Before 2010, there was no report on a mediating role of cGMP/PKG pathway in the biological effects of H_2S. Earlier studies showed that H_2S at physiologically relevant concentrations did not stimulate sGC (1). The activation of cGMP pathway was not involved in H_2S-induced relaxation of rat aortic tissues (311, 772), which was confirmed in rat coronary arteries (106).

Cellular cGMP level is reached at the balance of cGMP production and degradation. The activation of PDE isoenzymes is responsible for the hydrolysis of both cGMP and cAMP, therefore lowering cGMP and/or cAMP levels. H_2S may represent the first identified endogenous PDE inhibitor. Indirect evidence for this claim came from a study on tadalafil, a long-lasting inhibitor of PDE-5. Cardiac I/R induced by coronary artery ligation and release was lethal for the rats in this study, but the pretreatment of these animals with tadalafil rescued almost all of the ischemia/reperfusion rats as the infarct size of the hearts was significantly decreased (536). These cardioprotective effects of tadalafil could not be realized should the activities of PKG and CSE be inhibited. The critical role of CSE in this event was confirmed since the protective effect of tadalafil was diminished in CSE knockout mice. Direct evidence for tadalafil-induced myocardial H_2S production and PDE inhibition was obtained by Bucci et al. in 2010 (76). In their study, NaHS treatment of cultured rat aortic smooth muscle cells results in elevated cGMP levels. The role of endogenous H_2S in regulating cGMP level was confirmed by overexpression of CSE in these cells or silencing of CSE expression, which either increased or decreased, respectively, intracellular cGMP levels. Unfortunately, whether the activity of guanylyl cyclase was affected by H_2S was not tested. Since the presence of a nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) significantly reduced the ability of H_2S to enhance cGMP levels, it was believed that H_2S actually inhibited the breakdown of cGMP by inhibiting PDE. This believing was substantiated with a cell-free assay in which different semi-purified PDE isoforms were included. NaHS inhibited the activities of various PDE isoforms nonselectively in this assay, suppressing the hydrolysis of both cGMP and cAMP (76). **FIGURE 7** summa-

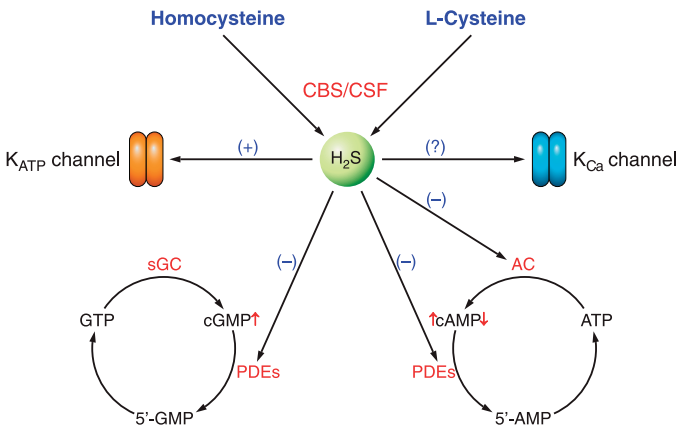


FIGURE 7. Different signaling pathways in cardiovascular system modulated by H_2S. AC, adenylyl cyclase; sGC, soluble guanylyl cyclase; PDEs, phosphodiesterases. The inhibitory effect is denoted with (−), and the stimulatory effect is denoted with (+).

rizes our current understanding of the effects of H_2S on cAMP and cGMP pathways.

D. MAPK Family

The MAPK superfamily is composed of three main members, stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK), p38-MAPK, and ERK. The activities of MAPK are critical in regulating cell proliferation, apoptosis, differentiation, inflammation, and cycle progression. MAPK can be differently affected by H_2S, leading to different cellular reactions.

1) The activation of different MAPK members by H_2S in different types of cells may lead to the same cellular events. H_2S induces apoptosis of human aortic SMCs via activating ERK (735, 737), but H_2S-induced apoptosis of INS-1E cells is mediated by the activation of p38 MAPK, not ERK (740). The mediating role of ERK was demonstrated by the time-dependent phosphorylation of ERK after NaHS treatment (735) and by a lower level of phosphorylated ERK in vascular tissues from CSE KO mice than in wide-type mice (737). Also, application of exogenous H_2S to cultured SMCs from CSE KO mice led to a greater phosphorylation of ERK than in SMCs from wide-type mice (737). The mediating role of p38 MARK in INS-1E cells was demonstrated as both exogenous H_2S (100 μM) and CSE overexpression inhibited ERK but activated p38 MAPK. Only inhibition of p38 MAPK, not ERK, decreased H_2S-stimulated apoptosis (740).

2) The activation of the same MAPK member by H_2S in different cells may be responsible for the opposite functional consequences. Again, let's look at the ERK pathway. H_2S increased endothelial cell proliferation via stimulating a sustained phosphorylation of ERK (467). The same increase in ERK phosphorylation by H_2S leads to inhibited proliferation of SMCs (735, 737). Upregulation of JNK expression by NaHS treatment (0.2–5 mM for 4 h) increased the proliferation of nontransformed intestinal epithelial cells (IEC-18) cells (141), but downregulation of JNK is responsible for the anti-apoptotic effect of NaHS on human derived dopaminergic neuroblastoma cell line (SH-SY5Y) (247).

3) H_2S-induced activation of the same MAPK member in different cells can induce the same cellular reactions. The inhibited phosphorylation of p38 was responsible for H_2S-induced survival of human polymorphonuclear cells (517) and anti-inflammation of microglial cells (248). It was also required for H_2S-induced survival of SH-SY5Y cells (247).

E. Cell Cycling Checking Points

Cell cycle is realized by the progression from G_1 phase to S phase, G_2 phase, and M phase (mitosis). Once this progression is stopped at any checking point, the cells enter G_0 phase or the so-called quiescence status. The fate of cells going through this cycle is determined at three checkpoints. The first checkpoint occurs at the end of G_1 phase, determined by the formation of protein complexes between cyclins and cyclin-dependent kinases (cdk). The association of cyclin D with cdk2/4/6 with cyclin D1 causes the cell progress to S phase. The functionality of Cdc25, a phosphatase, largely determines whether the cell can pass the second checking point to move on from G_2 phase to M phase. The third checking point involves the degradation of cyclin B in M phase, which is critical for sister chromatid separation.

H_2S can affect cell proliferation or death by altering the fate of the cell going through cell cycle. NaHS (1 mM) promoted the proliferation of nontransformed intestinal IEC-18 cell by facilitating the cell cycle entry (141). In oral epithelial-like cells, Ca9–22, H_2S (5 and 10 ng/ml) significantly decreased DNA synthesis. The proportion of cells in G_1 phase was significantly increased, and the proportion of cells in S phase decreased. H_2S treatment also resulted in a decreased Rb phosphorylation and increased p21Cip1. Takeuchi et al. (615) then concluded that the inhibitory effect of H_2S on the epithelial-like cells was due to cell cycle arrest via the expression of p21Cip1 (615). In all phases of the cell cycle, p21Cip/WAF-1 is involved in the control of cyclin-cdk activity. In isolated SMCs and vascular tissues from CSE KO mice, $p21^{Cip/WAF-1}$ level was decreased but cyclin D1 expression increased. Similarly, NaHS increased $p21^{Cip/WAF-1}$ expression and decreased cyclin D1 expression in SMCs from CSE-KO mice, but not in SMCs from wild-type mice. This study confirms and extends the observation on epithelial-like cells (615) that H_2S-induced cell cycle arrest involves the downregulation of cyclin D1 and upregulation of $p21^{Cip/WAF-1}$ (737).

F. ER Stress

The ER is where lipid synthesis and the mature and folding of membrane proteins, secretory proteins, and Golgi apparatus and lysosomes proteins occur. It also plays an important role in regulating intracellular signaling process, including calcium homeostasis. ER stress refers to altered ER function in general and to accumulation of unfolded protein aggregates or excessive protein traffic specifically.

A marked cardiomyocyte ER stress was produced in rats with HHcy either by homocysteine injection subcutaneously (98) or by methionine overloading (696). In both cases, HHCy was accompanied by reduced CSE expression and H_2S production in cardiac tissues. The expressions of ER stress-associated proteins, including C/EBP homologous protein (CHOP), glucose-regulated protein 78 (GRP78) and caspase-12 in myocardial tissues and lipid peroxidation were decreased by H_2S gas bubbled solution (intraperitoneal). In cultured H9c2 cells (rat embryonic heart-derived

cell line), the inhibition of endogenous H_2S production worsened cardiomyocyte ER stress and exogenous H_2S treatment ameliorated it, reflected by corresponding changes in the expression of CHOP, cleaved caspase-12, and p-eIF2alpha expressions induced by homocysteine.

ER stress in cardiomyocytes showed low endogenous H_2S production as discussed above, but high H_2S level results in ER stress in cultured INS-1E cells (740). Overexpression of CSE or application of exogenous H_2S caused INS-1E cell apoptosis as well as upregulated the expression of BiP and CHOP. Then, knocking down CHOP expression diminished H_2S-induced apoptosis of INS-1E cells. Moreover, H_2S-induced cell apoptosis and ER stress were suppressed by the inhibition of p38 MAPK. These results indicate that p38 MAPK activation functions upstream of ER stress to initiate H_2S-induced apoptosis (740).

G. Antioxidant and Reducing Capacity

H_2S is a strong reducing agent and may easily interact with other oxidative species. Its group allows the reduction of disulfide bonds and scavenging of reactive oxygen species and nitrogen species (701). Direct scavenge of peroxynitrite and the reduction of its toxicity by H_2S has been reported (700). This effect would endow H_2S an antioxidant role to offer cytoprotection (667). H_2S significantly inhibited $ONOO^-$-mediated toxicity and tyrosine nitration at concentrations well within the physiologically relevant levels (1, 306, 214, 700). Its antioxidant potency in terms of $ONOO^-$ scavenging is comparable to that of GSH (41, 704). Excessive $ONOO^-$ production (as 3-nitrotyrosine) is a trademark of various neurodegenerative diseases (38). On the other hand, GSH is present inside neurons and glia at millimolar concentrations, but hardly detectable in extracellular space (37). Due to its high membrane permeability, H_2S in the CNS would help reduce $ONOO^-$ level inside and outside cells, a target that cannot be achieved by GSH alone.

GSH redox cycle is a major endogenous protective system, with GSH being a major intracellular antioxidant. Depletion of cellular GSH results in the accumulation of reactive oxygen species and loss of mitochondrial function. The antioxidant property of H_2S may also be indirectly realized due to its capability in increasing the level of GSH. This could partially result from the stimulatory effect of H_2S on γ-glutamylcysteine synthetase and cysteine transport in neurons (310).

Previous studies have shown that the presence of superoxide dismutase and catalase did not change H_2S-induced vasorelaxation of rat vascular tissues (772, 773). These observations suggest that the vascular contractility change induced by H_2S may not be linked to a changed redox status of vascular tissues. But the remodeling or proliferative process of blood vessels can be affected by H_2S through its antioxidant effect (400, 685). H_2S (25–200 μM) treatment of isolated human aorta or its primary braches with atherosclerotic lesions reduced lipid hydroperoxide content of oxidized LDL and lipid extracts derived from soft atherosclerotic plaque (273). At the cellular level, homocysteine treatment of cultured vascular smooth muscle cells elevated cellular levels of H_2O_2, $ONOO^-$, and O_2^- and caused cytotoxicity. These adverse effects were counteracted by low levels of NaHS (30 or 50 μM) (730). Similarly, hydrogen peroxide and oxLDL-induced cytotoxicity of cultured HUVECs was reduced by H_2S (50 μM) treatment (273). The accumulation of lipid peroxidation products in HUVECs, including conjugated dienes, lipid hydroperoxides, and thiobarbituric acid reactive substances during hemin-mediated oxidation were also diminished (273).

H. Protein *S*-Sulfhydration

One of the most recently discovered signaling mechanisms for H_2S effect involves covalent modification of cysteine residues in proteins through *S*-sulfhydration, converting cysteine -SH groups to hydropersulfide (i.e., -SSH groups) (420). For a better understanding of its formation and wide biological implications, *S*-sulfhydration would have to be discussed in a comparative fashion with *S*-nitrosylation. *S*-nitrosylation occurs between NO molecules and cysteine residues of the concerned proteins (590, 591). Striking similarities between *S*-sulfhydration and *S*-nitrosylation have been noticed as both involved the covalent modification of cysteines and both are reversible by reducing agents, such as dithiothreitol (DTT) (420).

Significant differences between *S*-nitrosylation and *S*-sulfhydration have been identified.

1) The abundance of protein posttranslational modification is different. Approximately 10–25% of endogenous glyceraldehydes 3-phosphate dehydrogenase (GAPDH), β-tubulin, and actin are *S*-sulfhydrated in vivo. In contrast, *S*-nitrosylation may only be applicable to ~1–2% of targeted proteins (420).

2) The functional outcome of the protein modification is different. *S*-Sulfhydration usually contributes to the increased activity of the modified proteins, but *S*-nitrosylation appears to decrease it in most cases. The latter could be due to the shielding of critical reactive thiol groups of the proteins. Let's take GAPDH as an example. This enzyme in the glycolytic pathway contains 16 cysteine residues. It has been shown that GAPDH activity is significantly lower in CSE KO mice than in wide-type mice, and exogenous H_2S increases GAPDH activity. Overexpression of CSE in HEK293 cells activates GAPDH, further confirming the important role of endogenous H_2S production. Direct interaction of H_2S and GAPDH leads to a sevenfold increase in

GAPDH activity (420). On the other hand, incubating the purified GAPDH with NO modified its four cysteine residues per molecule, which abolished its catalytic activity (230).

3) *S*-Sulfhydration is more stable in comparison of *S*-nitrosylation. As such, the detection of *S*-sulfhydration can be relatively easy with mass spectrometry, but it is not the case with the more labile *S*-nitrosylation.

4) *S*-sulfhydration occurs under the resting conditions without the need of additional physiological stimulation. This is evidenced by the 25–30% reduction of liver GAPDH sulfhydration after CSE is knocked out and endogenous H_2S production is minimized despite normal levels of GAPDH protein (420). In contrast, *S*-nitrosylation of GAPDH is not affected in livers of neuronal NOS (nNOS), eNOS, and iNOS knockouts (230), indicating the basal level of NO would not have meaningful impact on GAPDH modification.

Distinction needs to be made between *S*-sulfhydration and *S*-thiolation (*S*-thionylation). The latter involves the formation of mixed disulfide between a protein thiol and a small-molecular-weight thiol such as glutathione or cysteine (637). The consequence of *S*-thiolation is the inactivation of the modified protein due to the blockade of the protein thiol. An increased protein function ensues after its *S*-sulfhydration, however.

Also, the prerequisite for *S*-sulfhydration is the availability of free -SH groups. If this condition cannot be met and the existence of disulfide bond barriers the access of H_2S to sulfhydryl groups, a two-step process would be involved. As a reducing agent, H_2S can disrupt disulfide bonds within proteins by acting as a reducing agent. This first step would help expose free SH groups. Step 2 will be *S*-sulfhydration. This may well be the case of the modification of SUR subunit of K_{ATP} channels by H_2S (279).

K_{ATP} channel complex is composed of two subunits. The pore-forming subunits (Kir6.x) conduit K^+, and SUR.x subunits are the binding sites for sulfonylurea to close Kir6.x and for MgADP or K^+ channel openers to open them. Jiang et al. (279) heterologously expressed Kir6.1 and SUR1 subunits in HEK-293 cells to form functional K_{ATP} channels. They subsequently demonstrated that the stimulatory effect of H_2S on K_{ATP} channel complex relied on a direct interaction between H_2S molecule and SUR subunits. The question then asked was where within the structure of SUR subunit is the target of H_2S. With the aid of mutagenic approach, it was eventually found that H_2S targeted at the disulfide bond between two cysteine residues, located on the extracelluar loop of the SUR1 subunit. Point mutation of either of these two (C6S and C26S) abolished the stimulatory effect of H_2S on K_{ATP} channels. The breakdown of the disulfide bond is therefore the first step for H_2S effect. It is likely that H_2S will then covalently sulfhydrate one or both these free -SH groups, which keeps the channel open. The hydropersulfide moiety (-SSH) can become an access point for agents to affect K_{ATP} channels. This could explain how *N*-ethylmaleimide (NEM) blocks K_{ATP} channels as this agent can only interact with free sulfhydryl groups or hydropersulfides **(FIGURE 8)**. The *S*-sulfhydration of Kir6.1 subunits heterologously expressed in HEK293 cells has also been reported based on the biotin switch assay result (420). The configuration and functional consequences of this sulfhydration on K_{ATP} channel currents wait for further exploration.

The challenges for the physiological importance of *S*-sulfhydration mechanism are still many. Given that *S*-sulfhydration is a relative stable modification, the fast and reversible effects of H_2S, such as the changed cardiovascular functions, need to be carefully analyzed. It is possible that some of these effects of H_2S may not be the results of *S*-sulfhydration. Another reasonable speculation would be the existence of endogenous desulfhydration mechanism. The regulation machineries for *S*-sulfhydration are also not clear. ATP level, pH value, ionic strength, and oxygen partial pressure may all affect *S*-sulfhydration process, but we do not know much about them. The reactive cysteine residues are the target of both nitrosylation and sulfhydration. Cys-150 of GAPDH can be nitrosylated (230) or sulfhydrated (420), for example. The interaction or competition between NO and H_2S on the same target would determine the eventual functional change of the concerned protein, but again, we do not have knowledge about this competition. The existence of endogenous desulfhydration molecules is intriguing. Phosphorylation of a protein is counteracted by dephosphorylation with phosphatase. *S*-nitrosylation can be reversed by denitrosylation with thioredoxins (44). As a hypothesis, H_2S itself may also function as a desulfhydration molecule due to its reducing property. This role may also be performed by other strong endogenous reducing molecules.

XI. H_2S AND ION CHANNELS

Ion channels are pore-forming membrane proteins that help establish and control the small voltage gradient across plasma membrane of cell or intracellular organelle membranes. The driving forces for the ion flows are membrane potentials and the ionic gradients for specific ions like Na^+, K^+, Ca^{2+}, and Cl^-, which allow for the flow of ions down their electrochemical gradient. Ion channels are generally classified into voltage-gated channels (e.g., L-type and T-type Ca^{2+} channels), ligand-gated channels (e.g., $TRPV_1$ and $TRPA_1$), and stretch-gated channels. These channels, individually or collectively, participate in the regulation of cell differentiation, muscle contractility, neurotransmitter release, or hormone secretion. This section reviews the interaction of H_2S with different types of ion channels and underlying molecular mechanisms.

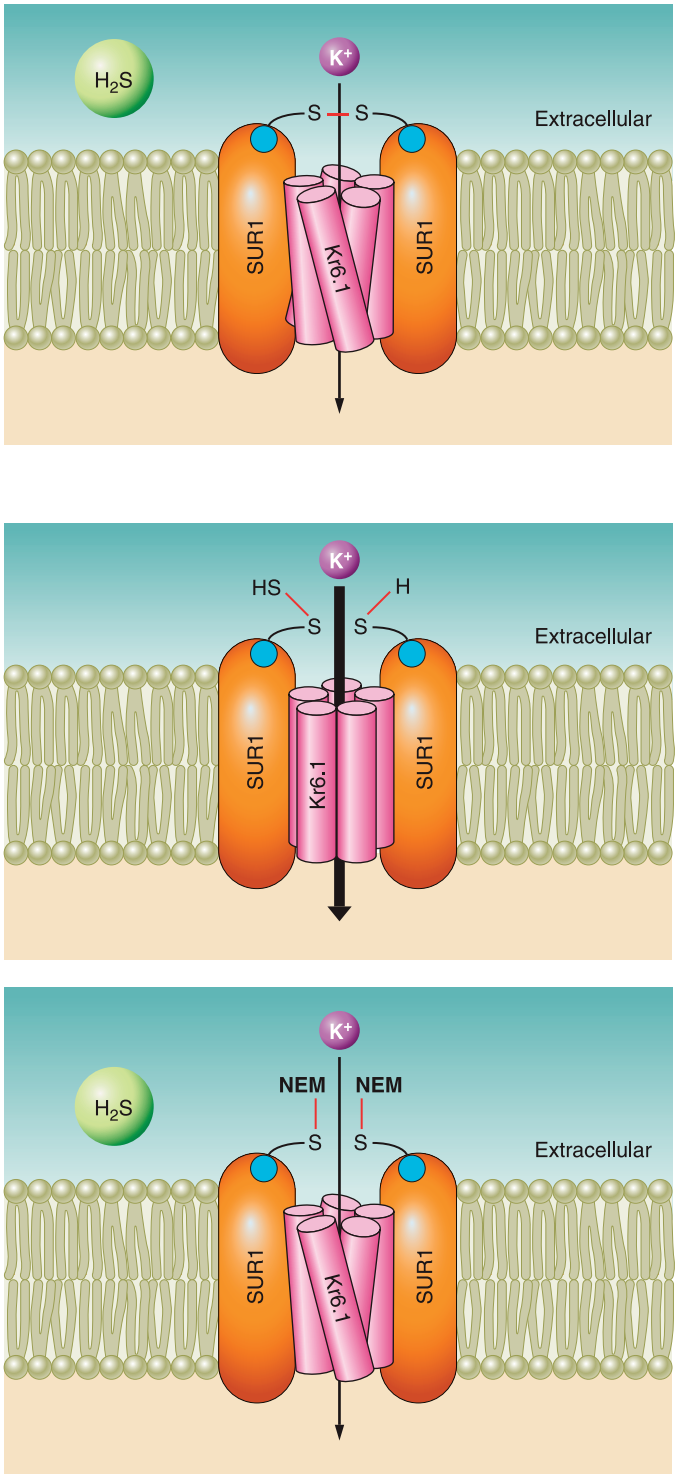


FIGURE 8. Schematic mechanism for the interaction of H_2S and *N*-ethylmaleimide (NEM) on cysteine residues of SUR subunit of K_{ATP} channel complex. This scheme is modeled based on the report of Jiang et al. (279).

A. K_{ATP} Channels

ATP-sensitive K^+ (K_{ATP}) channels are composed of pore-forming subunits (Kir6.x) and sulfonylurea receptor (SUR) subunits that couple cellular electrical activity to metabolism in a variety of tissues. By targeting K_{ATP} channels, H_2S regulates the processes of inflammation, nociception, pain, and cell death and exerts its beneficial protective effects against ischemia damage, hypertension, inflammation, nociceptiveness, and apoptosis, etc.

Extensive experiments on vascular tissues strongly suggest that H_2S-induced vasorelaxation is mainly caused by K_{ATP} channel openings. This notion is largely based on the ability of glibenclamide, a K_{ATP} channel antagonist, to block the vasorelaxant effects of H_2S (112, 773). Electrophysiological study provides direct evidence that exogenous H_2S increases macroscopic or unitary K_{ATP} currents, which is blocked by glibenclamide in isolated rat aortic and mesenteric SMCs (620, 773). H_2S-induced hyperpolarization of SMC membrane is also abolished by glibenclamide. In isolated piglet cerebral arteriole SMCs, a recent study showed that H_2S activated K_{ATP} channels at physiological steady-state voltage (−50 mV), which was antagonized by glibenclamide (370).

The opening of K_{ATP} channels in myocardium has been seen to play a pivotal role in cardioprotection during irreversible I/R injury, which is specifically seen in cardiac ischemic preconditioning (218). It was observed that in the perfused rat heart preparation, NaHS concentration-dependently limited the size of infarction induced by left coronary artery ligation, and this protective effect was abolished by K_{ATP} channel blockers glibenclamide and 5-hydroxydecanoate (284). Reperfusion of the isolated Langendorff-perfused heart with NaHS after ischemia attenuated arrhythmias and improved cardiac function during I/R. These effects of NaHS were blocked by glibenclamide, which suggests that H_2S produces a cardioprotective effect against I/R injury during reperfusion, at least in part by opening K_{ATP} channels (284). The patch-clamp data provide additional electrophysiological evidence that convincingly shows the effect of H_2S on K_{ATP} channels. Exposure of single cardiac myocytes to NaHS increased single-channel activity of K_{ATP} channels by increasing the open probability of these channels without altering single-channel conductance (769). This increase in the open probability can be blocked by glibenclamide. Therefore, the cardioprotective effect of H_2S involves not only the opening of K_{ATP} channels, but also the activation of cardiac ERK and/or Akt pathways in addition to preserving mitochondrial structure and function (160, 248).

H_2S-induced neuroprotection and suppression of glutamate toxicity was partially mediated by the activation of K_{ATP} channels. Glibenclamide and glipizide dose-dependently suppress H_2S-induced protection of HT22 cells from oxidative stress. Neuroprotection was increased by the simultaneous application of H_2S and pinacidil or the combined application of cysteine and pinacidil. While all these results support the involvement of plasma membrane K_{ATP} chan-

nels in the effects of H_2S, opening (with diazoxide) or blocking (with 5-hydroxydecanate, 5-HT) of mitochondrial K_{ATP} (mitoK_{ATP}) channels did not modulate protection by H_2S (309, 310).

Distrutti et al. (148) have demonstrated that the systemic administration of different H_2S donors inhibits visceral nociception by opening K_{ATP} channels. The activation of K_{ATP} channels in the peripheral nociceptive system has been seen to be involved in the modulation of nociception (579). For instance, peripheral antinociceptive drugs that directly block ongoing hypernociception induced by PGE_2, such as morphine and dipyrone, exert their effects by opening K_{ATP} channels stimulated by the NO-cGMP antinociceptive pathway (579). Cunha et al. (129) tested the hypothesis that the antinociceptive effect of H_2S on direct hypernociception induced by PGE_2 is dependent on K_{ATP} channels in the periphery. Supporting this hypothesis, glibenclamide prevented the antinociceptive effect of exogenous H_2S in rodents. A possible direct hypernociceptive effect of glibenclamide was excluded, as glibenclamide administration alone in the rat paw did not produce mechanical hypernociception (535). Local administration of a K_{ATP} channel opener also directly blocks hypernociception induced by PGE_2, which further supports the findings. Electrophysiologically, it has been shown that K_{ATP} channel activation reduces the enhanced excitability of rat nociceptive sensory neurons induced by PGE_2 (129).

A key event in inflammation is the recruitment of circulating leukocytes into the damaged tissue. Andruski et al. (10) used intravital fluorescence microscopy to look at leukocyte behavior in an intact rodent knee joint and later surmised that local treatment of acutely inflamed knee joints with an H_2S donor limited leukocyte recruitment and trafficking and decreased synovial blood flow. These anti-inflammatory effects of H_2S were mediated via the K_{ATP} channel because responses could be blocked by glibenclamide treatment. Intra-articular administration of NaHS had no effect on joint pain sensation or secondary allodynia in the rat, although this observation needs to be corroborated in other animal species.

Thus it is conceivable that H_2S may function as an endogenous regulator of joint function and that its action is distinctly anti-inflammatory (10). However, exogenously administered H_2S acts on sensitive neurons and promotes the opening of K_{ATP} channels and subsequent antinociception (129).

The effects of H_2S on K_{ATP} channels also exert influence on pain cognizance. Research has clarified that parenteral administration of either NaHS or an H_2S-releasing derivative of mesalamine inhibited dose-dependently visceral nociception in a colorectal distension (CRD) model in the rat. Administration of L-cysteine also reduced rectal sensitivity to CRD. The inhibitory effect of NaHS on CRD-induced pain or antinociception was completely reversed by pretreating rats with glibenclamide (148). Also, glibenclamide inhibited colonic smooth muscle relaxation induced by the highest dose of NaHS. The antinociceptive and muscle relaxant effects of NaHS were mimicked by pinacidil. These results show that H_2S functions as a negative regulator of visceral nociception by activating K_{ATP} channels and attenuating pain. NaHS-induced antinociceptive effects are not dependent on the activity of capsaicin-sensitive pathways that can induce smooth muscle contraction (472), although CRD-induced pain is closely related to increased contractility of colorectal smooth muscles. NaHS induced antinociception only at relatively low doses, but caused intestinal smooth muscle relaxation at high doses.

Due to the crucial role of K_{ATP} channels in the regulation of pancreatic insulin secretion, multiple studies have examined the effect of H_2S on β-cells. K_{ATP} currents were limited after lowering endogenous H_2S level in INS-1E cells, derived from rat insulinoma cell line, by CSE-targeted short interfering mRNA transfection, which was blocked by gliclazide and stimulated by diazoxide (741). Endogenously produced H_2S by overexpression of the CSE gene significantly aggrandized whole cell K_{ATP} currents in INS-1E cells. Exogenous H_2S markedly increased the open probability of single K_{ATP} channels by twofold in inside-out patches, but single-channel conductance and ATP sensitivity of K_{ATP} channels were not changed by H_2S (741). From a therapeutic point of view, pharmacological modulators of β-cell-type K_{ATP} channels could possibly be utilized to selectively target at the K_{ATP} channels in other metabolically sensitive cells that share the same molecular makeup of Kir6.2 and SUR1 subunits. The same strategy would also find its application in vascular tissues. The expression of Kir6.1 and SUR1 subunits in rat mesenteric arteries (279) and that of Kir6.1 and SUR2 subunits in piglet arterioles (370) have been reported. The vasorelaxant effects of H_2S in these vascular tissues are most likely mediated by the specific modification of SUR1 or SUR2 subunits.

B. K_{Ca} Channels

It has been observed that H_2S-induced vasorelaxation of rat aortic ring was not affected by iberiotoxin or charybdotoxin. This observation suggests that big-conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) channels might not be responsible for the H_2S-induced vasorelaxation in conduit vessels (773). Both H_2S and NaHS evoked concentration-dependent relaxation of in vitro perfused rat mesenteric artery beds (MAB) (112). The vascular effects of H_2S on MAB were related to the stimulation of charybdotoxin/apamin-sensitive K^+ channels in the vascular endothelium, in addition to the activation of K_{ATP} channels in vascular SMCs. Similarly, a combination of charybdotoxin and apamin abrogates the vasorelaxant effect of H_2S in the endothelium-

intact rat aorta. These data suggest that small to medium conductance K_{Ca} channel (SK_{Ca} and IK_{Ca}) in MAB and aorta is activated by H_2S. Therefore, H_2S might fulfill the role of EDHF (684). The stimulation of SK_{Ca} and IK_{Ca} channels by H_2S was also indirectly demonstrated in isolated rat mesenteric arteries as well as in isolated vascular endothelial cells, based on the changes in membrane potential (421).

One recent patch-clamp study showed that NaHS arrested heterologously expressed BK_{Ca} channels in HEK-293 cells transfected stably with human BK_{Ca} channel α-subunits (631). In contrast to the effects of CO donors, NaHS decreased the open probability and shifted the BK_{Ca} α-channel activation curve rightward without altering its conductance, suggesting that the action of H_2S and CO are noncompetitive. The same conclusion of H_2S-induced inhibition of BK_{Ca} channels was drawn in type I glomus cells of mouse carotid body (363). In sharp contrast, a recent report showed that NaHS augments whole cell BK_{Ca} currents and enhances single-channel BK_{Ca} activity in rat pituitary tumor cells (GH3) by increasing channel open probability (570). The above three patch-clamp studies used NaHS at the same concentration range (~300 μM), but the conclusions are opposite. No explanation has been given, but it might be related to specific BK_{Ca} channel subtypes in different types of cells (622). Another study by Jackson-Weaver et al. (271) examined the myogenic tone of rat mesenteric arteries and cerebral arteries as well as the membrane potential of vascular SMCs. Although the authors did not directly record changes in K_{Ca} channel currents, their results nevertheless showed that exogenous H_2S dilated and hyperpolarized rat arteries and that these effects of H_2S were blocked by iberiotoxin and paxillin. Thus the stimulation of iberiotoxin-sensitive BK_{Ca} channels by H_2S is suggested (271).

C. Cl^- Channels

The ATP-binding cassette superfamily includes cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels and sulfonylurea receptors, which are components of K_{ATP} channels. Both subunits also share key sequence homologies. The Cl^- channel blockers 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and indyanyl oxyacetic acid (IAA-9) suppress protection by H_2S, while levamisole, which is an opener of Cl^- channels, competently stops glutamate toxicity (309). This research purports that CFTR Cl^- channels may also be involved in protection by H_2S against oxidative stress. The recent findings that a decrease in transmembrane Cl^- gradients causes cell death in hippocampal pyramidal neurons and that the expression of CFTR gene is reduced in the hypothalamus of patients with AD (336) suggest that homeostasis of transmembrane Cl^- gradients is required for normal cell survival. Subsequently, the effect of H_2S on Cl^- channels in the CNS has been studied. In the research, H_2S was seen to initiate CFTR Cl^- channels in HT22 neuronal cell lines which led to neuroprotection during oxytosis. This was demonstrated through dose-dependent suppression of neuroprotection due to H_2S using specific CFTR blockers, NPPB and IAA-94, and confirmed using CFTR activator levamisole (309). Together with the recent observation of H_2S activating Cl^-/HCO_3^- transporters in smooth muscle cells (343), the results suggest possible regulation of Cl^- fluxes by H_2S in the CNS with neuroprotective consequences. The regulation of inhibitory Cl^- currents coincides with the regulation of inhibitory K^+ channels and therefore strongly purports a key role for H_2S in mediating excitability (622).

D. Ca^{2+} Channels

It is well recognized that voltage-activated Ca^{2+} channels (VDCC) regulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and consequently impact Ca^{2+} signaling in excitable cells. Ca^{2+} channels are classified, based on their electrophysiological features, as high voltage-activated (HVA) and low voltage-activated (LVA) types. The former include L-, N-, P-/Q-, and R-type channels, and the latter are actually T-type channels. In addition to Ca^{2+} channels in the membrane, $[Ca^{2+}]_i$ is controlled by intracellular Ca^{2+} stores. $[Ca^{2+}]_i$ changes due to extracellular Ca^{2+} entry may be facilitated by VDCC, transmitter-gated Ca^{2+}-permeant ion channels, transient receptor potential (TRP) ion channels, and Ca^{2+} pumps located in the plasma membrane (117). Channels that affect intracellular Ca^{2+} stores include ryanodine receptor (RyR) channels, inositol trisphosphate receptor (IP_3R) channels, and sarcoendoplasmic reticular Ca^{2+} ATPases (SERCA) (117).

1. L-type VDCC

A recent electrophysiological study characterized NaHS as an inhibitor of L-type VDCC in cardiomyocytes (606). NaHS caused a concentration-dependent decrease of the current density of whole cell VDCC and inhibited the recovery from depolarization-induced inactivation. NaHS did not alter the steady-state activation and inactivation curves. Additionally, bath application of NaHS significantly suppressed the shortening of single cardiomyocytes and contraction of isolated rat papillary muscles, which is associated with the inhibition of L-type VDCC by H_2S. Electric field-induced $[Ca^{2+}]_i$ transients in single cardiomyocyte were reduced by NaHS treatment (606). In cultured rat cerebellar granule neurons, NaHS raised $[Ca^{2+}]_i$ to the neurotoxic range and caused cell death, which were blocked by nifedipine and nimodipine, L-type VDCC antagonists. This finding supports the argument that NaHS activates L-type VDCC (202). However, no direct electrophysiological recording was conducted on these neurons. In astrocytes, Ca^{2+} waves induced by H_2S were found to be blocked by a selective L-type VDCC blocker nifedipine (427), which purports that the effect of H_2S on L-type VDCC is not

restricted to neurons alone. The body of evidence is suggestive that H_2S acts on either $Ca_v1.2$ or $Ca_v1.3$ L-type VDCC. Stimulation of Ca^{2+} entry via L-type VDCC by H_2S may promote processes such as neurotransmitter release and gene expression. A mounting amount of evidence has demonstrated that H_2S suppressed L-type VDCC and stimulated T-type VDCC, induced Ca^{2+} waves, and mobilized intracellular Ca^{2+} stores.

2. T-type VDCC

T-type VDCC also have critical roles to play in the processing of either somatic (479) or visceral (305) nociceptive information and in control of pain (301). Similar to capsaicin, NaHS, administered intracolonically, triggered visceral nociceptive behavior that was accompanied by referred abdominal hyperalgesia/allodynia (396). These responses are completely abolished by preadministered intraperitoneally mibefradil, a specific T-type VDCC blocker (396). In contrast, mibefradil at the same dose failed to attenuate the intracolonic capsaicin-induced visceral nociception. Neither L-type VDCC blocker verapamil nor K_{ATP} channel blocker glibenclamide modified the intracolonic NaHS-evoked visceral nociception. Furthermore, researchers found that intraperitoneal NaHS facilitated intracolonic capsaicin-evoked visceral nociception, which was also abolished by intraperitoneal pretreatment with mibefradil. Similarly, intraplantar administration of NaHS induced prompt mechanical hyperalgesia in rat hindpaw, which is blocked by mibefradil but not by glibenclamide (301). Therefore, H_2S likely functions as a novel nociceptive messenger through the activation of T-type VDCC during inflammation. Furthermore, PPG or BCA (CSE inhibitors) abolished the L-cysteine-induced hyperalgesia and attenuated the lipopolysaccharide-induced hyperalgesia, an effect being reversed by NaHS (301). Like the reducing agent dithiothreitol, NaHS increased T-type VDCC currents without alteration of their kinetics in undifferentiated NG108–15 cells, an effect being abolished by an oxidizing agent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). Suppression of T-type VDCC by DTNB at a high concentration was reversed by NaHS and dithiothreitol at subeffective concentrations.

T-type VDCC is also involved in pancreatic nociception in rodents (441). Either NaHS or capsaicin induced the expression of Fos protein in the superficial layers of the T8 and T9 spinal dorsal horn of rats or mice. The induction of Fos by NaHS but not capsaicin was abolished by mibefradil. In conscious mice, repeated doses of cerulein produced pancreatitis, accompanied by abdominal allodynia/hyperalgesia. Pretreatment with PPG prevented the allodynia/hyperalgesia, but not the pancreatitis. A single dose of mibefradil reversed the established pancreatitis-related allodynia/hyperalgesia. Taken together, H_2S appears to function as a novel nociceptive messenger through sensitization of T-type VDCC in the peripheral tissues, particularly during inflammation (622). The pronociceptive and antinociceptive effects of H_2S with the differential involvements of K_{ATP} channels and T-type VDCC have been discussed in more details in section VC.

In patch-clamp studies using undifferentiated NG108–15 cells, NaHS enhanced T-type VDCC currents, which may prove that H_2S activates these channels (301). These authors also reported that intraplantar (301, 386) and intrathecal (386) injections of NaHS promptly induced hyperalgesia in rats through T-type VDCC activation. Further investigation suggested that the $Ca_v3.2$ isoform of T channels was activated by H_2S, demonstrated by the abolishment of H_2S induced-hyperalgesia using a general T-type channel blocker mibefradil, and similar results were produced using $ZnCl_2$ ($Ca_v3.2$ specific inhibitor) and also with intrathecal administration of $Ca_v3.2$-specific antisense nucleotides to the rat (386). Using high (4.5–13.5 mM) concentrations of NaHS on undifferentiated NG108–15 cells, the same group was able to demonstrate that H_2S induced neurite outgrowth, which was found to be related to the activation of $Ca_v3.2$ isoform T-type channels demonstrated with the abolishment of neurite outgrowth using general T-type channel inhibitor mibefradil, intracellular Ca^{2+} chelator BAPTA-AM, and $Ca_v3.2$ isoform-specific blocker $ZnCl_2$ (428). Interestingly, they also discovered that H_2S induced high-voltage-activated Ca^{2+} currents that were composites of L-type, N-type, and P/Q-type channel activation (428). Therefore, by compiling the evidence by various authors, T-type channel activation, in particular the $Ca_v3.2$ isoform, by H_2S appears to regulate rhythmic neuronal activity, pain sensation, and differentiation of neurons and boosting of synaptic communication, similar to putative processes regulated by H_2S-related L-type channel activation.

E. TRP Channels

The mammalian TRP superfamily consists of 28 different proteins that may be subdivided into six main subfamilies. They are TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) (440). Several members that make up this protein superfamily have been found to be nonselective cation channels, of which many are located on primary sensory neurons and involved in somatosensory procedures, such as the transduction of chemical, thermal, and mechanical stimuli. $TRPV_1$ (also called capsaicin receptor) is a nonselective cation channel with high permeability of Ca^{2+} and activated by capsaicin and other vanilloid compounds. $TRPA_1$ is present on capsaicin-sensitive primary sensory neurons, which upon activation elicit pain, protective reflexes, and local release of neurotransmitters in the periphery (612).

1. $TRPV_1$

H_2S and its donors activate $TRPV_1$ ion channels in GI tract, airway, pancreas, and urinary bladder, which cause colonic mucosal Cl^- secretion, gut motility, airway constriction, acute pancreatitis, detrusor muscle contraction, and bladder contractility through a neurogenic inflammation mechanism (49, 472, 601, 647).

Serosal application of NaHS and L-cysteine stimulates luminal Cl^- secretion by guinea pig and human colonic tissues (542). This effect is blocked by TTX, desensitization of afferent nerves with capsaicin, or by the $TRPV_1$ antagonist capsazepine. As such, the stimulatory effects of H_2S on TTX-sensitive Na^+ channels as well as $TRPV_1$ channels are theorized (622). Interestingly, the secretory effect of NaHS is not observed in a human colonic epithelial cell line (T84 cells) (542). It appears that H_2S-stimulated mucosal secretion cannot be realized in the absence of either TTX-sensitive Na^+ channels and/or $TRPV_1$ channels from sensory nerve endings. In addition, NaHS-induced Cl^- secretion in rat distal colon is inhibited by serosally applied glibenclamide and tetrapentylammonium, suggesting the involvement of different types of K^+ channels (K_{ATP} and K_{Ca}) (234). As glibenclamide may inhibit CFTR, this result could also be interpreted as the direct activation of CFTR by H_2S to increase Cl^- secretion.

Similar to capsaicin, H_2S donors induce CGRP and substance P release from the sensory nerves in the guinea pig airways and cause in vivo bronchoconstriction and microvascular leakage in a capsazepine-sensitive manner. This adds to the irritant action of H_2S in the respiratory system (647). It has been found that NaHS induces a dose-dependent contraction of isolated bronchial and tracheal rings in vitro, and this effect is denigrated by the desensitization of sensory nerves with high concentration of capsaicin, by $TRPV_1$ antagonists (capsazepine and ruthenium red), as well as by a mixture of neurokinin NK1 (a substance P receptor) and NK2 receptor (CGRP receptor) antagonists. Interestingly, intraperitoneal injection of NaHS to healthy mice induced substantial inflammatory reaction in the lung, as evidenced by increased concentration of substance P, pro-inflammatory cytokines, TNF-α and IL-1β, and lung MPO activity (51). These effects were abolished by a specific NK1 receptor antagonist, but not by NK2 receptor antagonists. In addition, the inflammatory effect of H_2S was abolished by capsazepine and was not observed in mice lacking substance P and neurokinin-A due to the knockout of their common precursor gene, preprotachykinin-A (51). These data indicated that H_2S per se may induce neurogenic inflammation, even in the absence of other, often harmful, elements. Further research is still required to solve whether H_2S acts as an endogenous ligand of $TRPV_1$ or not (622).

Activation of $TRPV_1$ has been reported to mediate neurogenic inflammation in cerulein-evoked pancreatitis (257). Intravenous injection of the $TRPV_1$ agonist capsaicin activated a dose-dependent increase in Evan blue aggregation in the rat pancreas. This effect was halted by the pretreatment with the $TRPV_1$ antagonist capsazepine or the neurokinin-1 receptor antagonist CP96,345. Capsazepine also limited cerulein-induced Evans blue, MPO, and histological severity of inflammation in the pancreas, but no effect was seen on serum amylase (257). Consequently, enhanced plasma H_2S levels have recently been demonstrated in cerulein-induced pancreatitis (49), and administration of PPG reduces the morphological changes in acute pancreatitis, which consists mainly of edema, inflammation, and acinar cell injury/necrosis.

In contrast to its vasorelaxant effect, NaHS actually created concentration-dependent contractile responses in the detrusor muscle of the rat urinary bladder (471). This response generated rapid and persistent tachyphylaxis similar to the responses of capsaicin. However, this cannot be seen as a direct effect of H_2S on the muscle because it was destroyed by the combination of NK1 and NK2 receptor-selective antagonists as well as by high-capsaicin pretreatment, which could desensitize capsaicin-sensitive primary afferent neurons. The response to NaHS is mostly resistant to TTX, as is the effect of capsaicin in this organ. The results may be able to provide pharmacological proof that H_2S stimulates capsaicin-sensitive primary afferent nerve terminals with the consequent release of tachykinins, which subsequently produces contractile responses of the detrusor muscle. Furthermore, ruthenium red, a nonspecific blocker of $TRPV_1$ channels, blocked the H_2S-induced contractile response (472), but $TRPV_1$-selective antagonist capsazepine and SB366791 failed to do so. It has also been theorized that H_2S may stimulate the $TRPV_1$ receptor by a different way from those known activators.

2. $TRPA_1$

$TRPA_1$ is activated by a variety of plant-derived and environmental irritants, such as allyl isothiocyanate (AI), cinnamaldehyde (CA), allicin, and acrolein, all of which interact with cysteine residues in the ion channel proteins (384). Interestingly, acrolein and similar aldehydes are formed endogenously during inflammation. $TRPA_1$ was initially characterized as a noxious cold receptor (600), and lately its role in mechanosensation has been suggested (333, 646). In the rat bladder, $TRPA_1$ is expressed in unmyelinated sensory nerve fibers with similar pattern to that of $TRPV_1$. Interestingly, $TRPA_1$ is also present in the urothelium, detected at both transcriptional and protein levels. The stimulation of $TRPA_1$ channels induced detrusor overactivity. $TRPA_1$ appears to be consistently colocalized with $TRPV_1$ in the bladder afferents, which suggests a role of $TRPA_1$ in bladder chemosensation and mechanotransduction (601). Following pretreatment with protamine sulfate, NaHS increased maximal bladder pressure and reduced voided and infused volumes. NaHS evoked a time- and concentration-depen-

dent increase in $[Ca^{2+}]_i$ in Chinese hamster ovary cells expressing mouse or human $TRPA_1$, but not in untransfected cells. This indirect evidence for the activation of $TRPA_1$ by H_2S needs to be validated with more direct electrophysiologial recording. Should this role be confirmed, H_2S may function as a $TRPA_1$ activator potentially involved in inflammatory bladder disease and in lower urinary tract infection. Furthermore, bacterial metabolite H_2S induced by potential pathogens such as *Escherichia coli* (45) might activate $TRPA_1$ in lower urinary tract infections.

The interactions of H_2S with different ion channels are summarized in **TABLE 1**. H_2S is the first identified gaseous opener of K_{ATP} channels in vascular SMCs and regulates vascular tone by relaxing smooth muscle cells. In the heart, H_2S and its donors cause the negative inotropic and chronotropic action through activating sarcK_{ATP} and mitoK_{ATP} channels and inhibiting L-type Ca^{2+} channel activity, and exert cardioprotection during I/R injury. H_2S-induced reduction of blood pressure can be related to the activation of peripheral K_{ATP} channels in resistant vessel SMCs. The regulation of insulin secretion from pancreatic β cells by H_2S is via enhancing K_{ATP} channel and suppressing L-type Ca^{2+} channel activities. By elevating $[Ca^{2+}]_i$, H_2S may mediate glutamate-induced neurotoxity and neuronal cell death, but conflicting reports describe the protective effect of H_2S on neuron cells from oxidative glutamate toxicity by activating K_{ATP} and Cl^- channels. H_2S-induced hyperalgesia in the colon seems to depend on the sensitization of T-type Ca^{2+} channels. On the other hand, H_2S has a pronociceptive role through evoking the excitation of capsaicin-sensitive $TRPV_1$-containing sensory neurons. H_2S and its donors also activate $TRPV_1$ and $TRPA_1$ channels in nonvascular smooth muscle such as urinary bladder, airways, and GI tract, regulating smooth muscle contractility.

The opening of K_{ATP} channels by H_2S has been confirmed in cardiovascular, endocrine, and nervous systems, which constitute a major molecular mechanism for many cellular effects of H_2S. However, the molecular interaction of this gasotransmitter with K_{ATP} channel complex has not been clear and the relative contribution of cysteine sulfhydration in K_{ATP} channel proteins by H_2S merits further investigation. The effects of H_2S on voltage-dependent L-type Ca^{2+} channels or BK_{Ca} channels are inconclusive. Our current knowledge on the effects of H_2S on other ion channels such as T-type Ca^{2+} channels, $TRPV_1$, TTX-sensitive Na^+ channels, and Cl^- channels are mostly based on pharmacological assays and the direct measurement of ionic currents by patch-clamp techniques is needed. Future studies should examine the effects of H_2S on different types of ion channels in various types of tissues and cells with the aid of multidisciplinary approaches including molecular biology, gene mutagenesis, electrophysiology, and pharmacology. Altered effects of H_2S on ion channels under different pathophysiological conditions also call for intensive investigation.

XII. INTERACTION OF H_2S AND OTHER GASOTRANSMITTERS

H_2S, NO, and CO are all classified as gasotransmitters (681). The shared chemical and biological properties endow them with common molecular targets and similar cellular actions. They are compensatory to each other in regulating biological functions. For example, at the tissue level, all three are capable of inducing vasorelaxation. At the cellular level, all can inhibit oxidative phosphorylation with both H_2S and NO acting on cytochrome oxidase. They also compete with each other. Hemoglobin is a common "sink" for NO, CO, and H_2S (681). The occupation of the binding sites on hemoglobin by one gasotransmitter will affect the binding of others and as such alter their biological effects. However, the binding affinities of gasotransmitters are different. NO has the highest affinity to heme in the active site of sGC, increasing its activity. The binding affinity of CO to the heme of sGC is much weaker than NO without the assistance of other endogenous substances (190). Although H_2S interacts with many heme proteins such as cytochrome *c* oxidase, hemoglobin, and myoglobin (509), current knowledge tells that H_2S does not have direct interaction with sGC (see sect. X), which leads to the question of the affinity of this gasotransmitter to the heme in sGC. Even when eliciting the same cellular changes, not all gasotransmitters necessarily act on the same molecular targets. All three gasotransmitters facilitate LTP, but only H_2S, not NO or CO, stimulates NMDA receptors (1). At times, they antagonize each other to provide a counterbalance. NO is a free radical, although almost nonreactive. Its quick reaction with molecular oxygen or superoxide forms more active and harmful free radicals in dinitrotrioxide (N_2O_3) and peroxynitrite ($ONOO^-$), respectively. Peroxynitrite is a true active free radical. CO is relatively inert by itself without being directly involved in redox reactions. H_2S, on the other hand, is an antioxidant as opposition to the prooxidant role of NO. Another example is that NO and CO stimulate BK_{Ca} channels (714) while H_2S inhibits it (631). The interaction among gasotransmitters as a regulatory network is of essential importance for our understanding of numerous physiological and pathophysiological processes in our body **(FIGURE 9)**.

A. H_2S and NO

1. *Functional Interaction: Synergistic Versus Antagonistic Effects*

Although H_2S or NO alone relaxes vascular tissues, the integrated vascular effects of the two gasotransmitters are quite complex. The vasorelaxant effect of SNP, a NO do-

Table 1. *Modulation of ion channels by H_2S*

Ion Channel	Action	Functional Consequence	Reference Nos.
		K_{ATP} channels	
Vascular smooth muscle cells	(+)	Vasorelaxation	112, 620, 773
Cardiomyocytes	(+)	Negative ionotropic effect	206, 284, 769
Sinoatrial node pacemaker cells	(+)	Negative chronotropic effect	726
Pancreatic β-cells	(+)	Inhibited insulin secretion	741
Cortical neurons	(+)	Inhibited neuronal excitability	309
Adjacent dorsomedial hypothalamic neurons	(+)	Lowering blood pressure	139
Colonic smooth muscle	(+)	Antinociceptive effect	147, 148
Rat hindpaw (carrageenan-induced edema)	(+)	Anti-inflammatory effect	180, 755
HEK-293 cells transfected with rvKir6.1/rvSUR$_1$	(+)		279
		K_{Ca} channels	
Vascular smooth muscle	(+)	Vasorelaxation	112, 773
HEK-293 cells transfected with BK_{Ca} α-subunits	(−)	Mediating oxygen sensing	631
Type I glomus cells of mouse carotid body	(−)	Function of chemoreceptors	363
Rat pituitary tumor cells (GH_3)	(+)		570
		L-type Ca^{2+} channels	
Cardiomyocytes	(−)	Reduced cardiac contractility	606
Cerebellar granule neurons	(+)	Causing cell death	202, 223
Astrocytes and brain slices	(+)	Induced Ca^{2+} wave	427
Microglia	(+)	Induced Ca^{2+} wave	344
		T-type Ca^{2+} channels	
Rat hindpaw	(+)	Pronociception (hyperalgesia), neuronal outgrowth, and differentiation	396
DRG neurons and NG108-15 cells	(+)		301
		$TRPV_1$ channels	
Urine bladder	(+)	Contraction of detrusor muscle for bladder urination	471, 472
Airways	(+)	Airway constriction	51, 647
Colon	(+)	Mucosal Cl^- secretion	51, 344
Pancreas	(+)	Acute pancreatitis	49, 257
		$TRPA_1$ channels	
Urine bladder	(+)	Detrusor overactivity for bladder chemosensation and mechanotransduction	601
		Cl^- channels	
Hippocampal nerve cell line (HT_{22} cells)	(+)	Inhibited glutamate toxicity and protected oxytosis	309, 310
Cardiomyocyte membrane preparation	(−)	Cardioprotection from ischemia- reperfusion injury	387

Action refers to stimulation (+) or inhibition (−).

nor, on rat aorta was decreased by H_2S at 60 μM (773). This may be related to the reaction of NO with thiols. Thus formed *S*-nitrosothiols would temporarily store and transport NO (504). The immediate consequence of this reaction would be the lowered active NO level and reduced NO effect. The long-term effect, however, would be the availability of NO buffer so that NO can be released under other conditions. An earlier study showed that the NO-induced

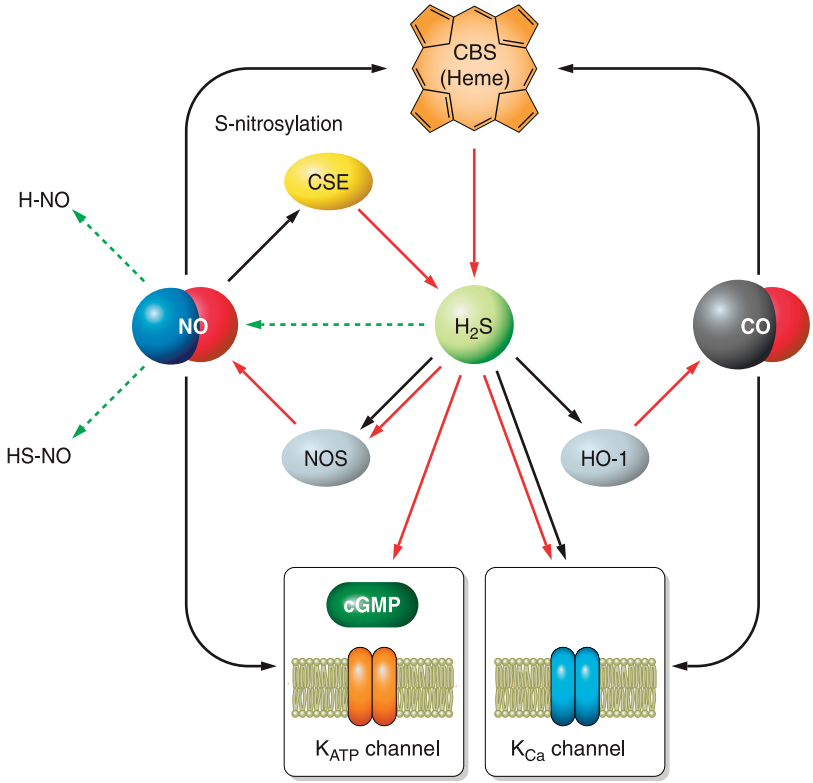


FIGURE 9. Interactions among 3 gasotransmitters: NO, CO, and H_2S. The red solid lines denote the stimulatory effect, and the black solid lines represent the inhibitory effect. The putative molecule-to-molecule interaction between H_2S and NO is denoted with the dotted lines.

relaxation of rabbit aorta and the increase in cGMP level were inhibited by L-cysteine and L-homocysteine (357). As both L-cysteine and L-homocysteine are endogenous precursors of H_2S and since L-cysteine also relaxed vascular tissues (112, 345) and nonvascular tissues (562), this study also lends support to an inhibitory effect of H_2S on the vascular effect of NO.

In contrast to the antagonistic effects of H_2S and NO described above, Hosoki et al. (245) reported the additive effects of SNP and NaHS (30 μM) in relaxing rat aortic tissues. This discrepancy may be partially explained by different experimental conditions used. The contractile stimuli to precontract vascular tissues, the concentrations of SNP and H_2S, and other factors may all contribute to the final outcome of the interaction of NO and H_2S. Hosoki et al. (245) used norepinephrine (1 μM)-precontracted helical tissue strips of aorta from Wistar rats, while Zhao et al. (773) used phenylephrine (0.3 μM)-precontracted aortic rings from Sprague-Dawley rats. The tissue damage of helical strips is greater than that of ring preparations. Also, the maximal contraction could be induced by 1 μM norepinephrine, while 0.3 μM phenylephrine, a submaximal concentration, only induced ~90% of the maximal contraction of rat aortic tissues. The advantage of using a submaximal concentration of phenylephrine is that the tissues can react with the relaxant agent in a more sensitive way. The take home message for this comparison would be that the integrated vascular effect of H_2S and NO is far more complex than a simple algebraic summation of their individual actions.

A synergistic inhibitory effect between NaHS and SNP was suggested in electrically stimulated guinea pig ileum tissues as the muscle relaxation was greater when both SNP and NaHS were present than with only one of them (630).

2. Effects of NO on H_2S Production

An earlier study showed that treatment of cultured vascular SMCs with NO donor increased the transcriptional level of CSE. While this effect provides an elevated enzymatic basis for long-term H_2S production, direct stimulation of CSE by NO to produce more H_2S provides an instant action (773). The latter was demonstrated after the homogenized rat vascular tissues were incubated with different concentrations of SNP and the accumulated H_2S in reaction mixture were measured. It may be argued that SNP may have other nonspecific effects not related to NO release. More evidence for the stimulatory effect of NO on H_2S production in rat aortic tissues has also been provided by the use of another NO donor, 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (771).

Can NO directly alter CSE activity? We do not have an answer for it yet. CSE is not a heme protein and as such an

analog cannot be used for the effect of NO on sGC. However, CSE can be the target of *S*-nitrosylation at its multiple reactive cysteine residues. Rat mesenteric artery CSE protein is an example as it contains 12 cysteines. Evidence for a mediating role of sGC/cGMP in the interaction of NO and H_2S, however, has been available that the blockade of PKG abolished the NO-induced increase in H_2S level in vascular tissues (771).

The NO-induced inhibition of H_2S production has been suggested. In liver, LPS-induced increase in H_2S production and CSE expression was inhibited by nitroflurbiprofen, a NO donor (11). This may be interpreted as a direct "cross-talk" between NO and H_2S synthesis or as an indirect outcome due to the suppression of inflammation by the NO donor, which thereafter removes the initial stimulation for H_2S synthesis. Unfortunately, it is not clear whether the administration of nitroflurbiprofen (intraperitoneal) could affect H_2S synthesis without LPS pretreatment.

3. Effects of H_2S on NO Production

LPS-increased NO production and iNOS expression were inhibited by exogenous H_2S at noncytotoxic levels in RAW264.7 macrophages (451). Administration of L-cysteine to boost endogenous H_2S production has the same inhibitory effects on NO and iNOS, but inhibition of CSE expression increased NO production. H_2S treatment of the same cells also increased HO-1 expression and CO production through the activation of ERK. But this is not a mere coincidence. The application of tin protoporphyrin IX to block HO activity or siRNA to knockdown HO-1 expression abolished the inhibitory effects of H_2S on iNOS expression and NO production. The application of CO mimicked the inhibitory effects of H_2S in terms of NO production and iNOS expression. Therefore, it was suggested that H_2S directly increases CO production and HO-1 expression, through which to indirectly inhibit NO production and iNOS expression.

Direct interaction of H_2S with NO synthases was reported under refined conditions. The activity of recombinant nNOS, iNOS, and eNOS was inhibited by NaHS with IC_{50} values of 0.13–0.21 mM in vitro. The absence of H_2S-generating enzyme in the reaction milieu rendered the inability of L-cysteine to affect the activities of these three NOS isoforms. As such, whether this inhibitory effect of H_2S on NOS isoforms can be realized under physiological conditions merits further investigation (327).

With the cultured cerebral vascular SMCs, but not endothelial cells, from pig brain, NaHS increased NO level by sevenfold (85). L-Cysteine incubation of these cultured cells increased NO level threefold in both SMC and EC, which was not affected by inhibition of CSE (85). Since endogenous H_2S production was not measured with L-cysteine treatment, it would still be premature to conclude the role of endogenous H_2S in the stimulation of NO production. In another study, using an intravital microscopic technique, Yusof et al. (753) showed that NaHS treatment for 24 h before I/R offered protection on leukocyte rolling, and this effect was largely abolished after eNOS inhibition or knockdown. NaHS treatment also reduced leukocyte adhesion, which was subsequently attenuated after eNOS inhibition. The authors suspected the involvement of an eNOS phosphorylation by NaHS (753).

NaHS may also facilitate the release of NO from its binding moieties (632). With the use of an electron paramagnetic resonance spectroscopy method of spin trap and by measuring the NO oxidation product, it was found that NaHS released NO from nitrosothiols (including nitrosoglutathione and SNAP), and from metal nitrosyl complex SNP (450). After rat brain homogenate or murine L1210 leukemia cells were incubated with NaHS, increased NO release was also detected. As these releases are more favored by pH 8.0 than pH 6.0, the contribution of HS^-, rather than H_2S, is suggested.

4. Molecule-to-Molecule Interaction Between H_2S and NO

The interaction of NO and thiol molecules is the base for NO-induced *S*-nitrosylation. H_2S is the simplest thiol molecule. Thus it is possible that H_2S interacts with NO to form nitrothiols (RSNO) **(FIGURE 9)**. Whiteman et al. (705) incubated NaHS with either NO donors or authentic NO gas in vitro. The time-dependent release of NO_2 from the reaction mixture indicated the formation of nitrosothiol. Electron paramagnetic resonance analysis revealed a spectrum characteristic of $[Fe(CNO_3)NO]_3$ with 30 s of mixing SNP and NaHS. These in vitro test tube results were echoed by tissue studies by the same authors (705). Adding NaHS to liver homogenates from LPS-treated rats increased NO_2 formation as an outcome of the interaction between exogenous NaHS and engogenous NO. Likewise, incubating liver homogenates with L-cysteine and pyridoxal phosphate to increase endogenous production of H_2S also led to increased NO_2 formation. The NO_2 formation under these conditions was especially elevated with an $HgCl_2$ pretreatment. Unlike authentic NO which stimulates cGMP production, this novel nitrosothiol would not be able to do so unless NO was released with Cu^{2+} (705).

In addition to RSNO, the formation of other moecules from the interaction of NO and H_2S has also been suggested. The putative reducing capacity of H_2S may generate nitroxyl (HNO), the one-electron reduced and protonated sibling of NO (746). Exposing isolated ventricular myocytes from adult rats to SNP or DEA/NO alone, but not to NaHS alone, led to decreased myocyte twitch amplitudes, slower rates of cell contraction and relaxation, lowered resting calcium level, and electrically induced calcium transient. These NO-elicited effects were reversed by NaHS (50 μM)

when this H_2S donor was given simultaneously with NO donors (746). The inhibition of NO effects by NaHS is not due to H_2S-induced alteration in target responses to NO. When the researchers mixed SNP and NaHS solutions, they found that this mixture duplicated the cellular effects observed with sequential applications of SNP and NaHS to the ventricular myocytes. The novel molecule responsible for the SNP+NaHS mixture was further zoomed in as a nitroxyl anion because Angeli's salt, an HNO donor, mimicked the effect of SNP + NaHS mixture on calcium handling and myocyte contractility (746). It should be cautioned to extrapolate the chemical nature of one molecule to another simply because the two molecules produce similar biological responses. Indeed, the researchers tried to scavenge HNO generated from the putative NO and H_2S interaction with *N*-acetyl-cysteine, L-cysteine, and glutathione. But these three thiols have much more cellular targets to act and more cellular functions to elicit than simply scavenging HNO. Moreover, the interaction of SNP and NaHS is not precisely equivalent to that of NO and H_2S.

The chemical nature of the novel compound(s) formed from the direct interaction of H_2S and NO cannot be precisely determined yet. The generation of HS-NO (705) or HNO (746) or other compounds will need to be verified chemically and analytically. The biological feasibility for the direct chemical interaction between H_2S and NO in vivo and its physiological importance also remain to be determined.

B. H_2S and CO

As discussed above, H_2S increased HO-1 expression and CO production in macrophages (451). Earlier studies also found that administration of H_2S to rats with hypoxic pulmonary hypertension increased plasma CO concentration and HO-1 expression in pulmonary artery (501).

Inhibition of HO activity with zinc protoporphyrin (ZnPP) markedly increased H_2S production and CSE expression in cultured aortic SMCs. Inhibition of CSE activity with PPG increased, but NaHS treatment (10–100 μM) decreased, CO production reflected by the increased HbCO level in the culture medium, and HO-1 expression in cultured cells (281). H_2S inhibits CO production and CO inhibits H_2S production, and the continuation of this cycle would leave no CO and no H_2S in the cells. Perplexing? There must be a checking point in vivo to interrupt this cycle, and the threshold concentration of either CO or H_2S may be the key under physiological conditions.

Finally, CO and H_2S can act on the same target but have opposite effects. While CO stimulated BK_{Ca} channels heterologously expressed in HEK293 cells, NaHS inhibited the channel with an IC_{50} of 670 μM (631). The physiological meaning of the effect of NaHS on BK_{Ca} channels at this high concentration aside, the actions of H_2S and CO were noncompetitive. This conclusion was derived from the observation that 1 mM KCN completely suppressed CO-evoked channel activation, but did not affect H_2S-induced channel inhibition (631).

C. CBS, a Heme-Containing Protein, Is a Target of NO and CO

Given that CBS is a heme-containing protein (399) and heme-containing proteins are common targets of NO and CO, the activity of CBS might be affected by NO or CO (73). CO can bind to the heme of CBS at its reduced state with different binding affinities (625, 626). CO binding to CBS may occur under physiological conditions as the association constants of CO binding to human CBS are 1.5 and 68 μM (498) and the physiological concentration range of CO is believed to be ~3–30 μM (264, 666). The binding of CO to CBS would inactivate CBC (559, 626). Furthermore, HO-1 or HO-2 and CBS are often colocalized in the same cell so that the endogenously generated CO can gain access to CBS in close proximity. Hepatocytes are the example for this colocalization (210, 292).

As of yet, the functional consequence of CO binding to CBS has not been clear. Since both CO and H_2S are involved in hippocampal LTP, by inhibiting CBS-based H_2S production in the CNS, CO can integrate the neuronal responses to different gasotransmitters. With an elevated endogenous CO level, other CO-sensitive cellular functions can be regulated by the inhibited CBS activity and lowered H_2S production. A case in point, the overproduction of CO in animal livers decreased H_2S production and lowered the levels of cystathionine, cysteine, and hypotaurine. Once the CBS gene was heterozygously knocked out, overproduction of CO did not affect these products of reverse transsulfuration pathway. This may suggest the contribution of CO inhibition to CBS activity in vivo, although it is not conclusive. Application of CO-releasing compounds to the rats decreased in hepatic H_2S content and stimulated HCO_3^--dependent biliary choleresis, which was not observed in CBS heterozygous knockout mice (559). In short, CBS may function as a CO sensor to coordinate the interaction of CO and H_2S.

The binding affinity of NO to CBS is lower than that of CO. Whether NO binding to CBS affects CBS activity has been controversial. Some studies showed decreased CBS activity due to NO binding (625), while other studies showed no change (559). The K_i value for NO is 200 mM, which is in sharp contrast to that of CO around 5 mM (625), suggesting that CBS senses CO rather than NO in vivo under physiological conditions. Another difference between CO and NO on their binding to recombinant CBS is that CO binds to the prosthetic heme and stabilizes 6-coordinated CO-Fe(II)-histidine complex. Also binding to heme, NO equalizes the five-coordinated structure (559).

XIII. DETECTION OF ENDOGENOUS H_2S PRODUCTION

A. Physiological Level of Endogenous H_2S

The determination of the physiological concentrations of H_2S in circulation and in specific tissues is pivotal for determining the impact of H_2S on a given physiological function; correlating H_2S levels with the specific pathophysiolgical changes; examining physiological roles of H_2S under in vitro conditions at organ, tissue, and cellular levels; and guiding pharmacological and therapeutic administrations of H_2S donors.

The physiological range of H_2S in circulation has been estimated at 10–100 μM in health animals and humans (243, 260, 514, 773). Aging appears to have no effect on circulating H_2S. A study revealed no change in serum H_2S concentration among three age groups of humans spanning 50–80 years (34–36 μM) (110). Rat serum contains 46 μM H_2S (773), and it is 34 μM in mouse serum (360). In New Zealand rabbits, a quantitative assay detects a plasma H_2S level around 16.5 μM (589). Plasma H_2S at micromolar ranges has also been reported in many other vertebrates (453).

Endogenous levels of H_2S in rat brain homogenates are 50–160 μM (1, 39, 213, 691). Similar H_2S levels were reported in the liver, kidney, and pancreas (213, 691, 754). H_2S production was clearly measured in the cardiovascular system (245, 773).

Not always the measurement of H_2S gives the consistent values. Using HPLC analysis, Sparatore et al. (584) reported a plasma sulfide level below 0.55 μM. Another study could not detect H_2S levels in lamprey, trout, mouse, rat, pig, and cow blood samples using a special house-made polarographic H_2S sensor that can detect 14 nM H_2S (708). One explanation for these low values of H_2S is the rapid decay of H_2S concentration from micromolar concentration to undetectable level within 30 min in vitro (247). Whether H_2S would disappear that fast in vivo is unknown. Regardless, even 30 min would be far more than sufficient to regulate a specific physiological function (150). A quick decay may actually indicate a homeostatic mechanism to trigger and to end H_2S signaling. Another related concern is the measurement technologies themselves. The real-time polarographic sensor was initially developed by Doeller et al. in 2005 (150). Using the same kind of sensor, Benavides et al. (43) demonstrated that red blood cells produced H_2S. In two other studies using polarographic sensors, free H_2S concentrations in whole rat blood have been detected at $\geq$5 μM (150, 313). As the polarographic sensors are house-made in the study by Whitfield et al. (708), whether the failure to detect H_2S in animal blood was due to some intrinsic factors with the sensor itself cannot be commented on. Availability of these house-made sensors to other research teams would have helped replicate these results or allowed for a better comparison. The simultaneous employment of the polarographic sensor and other detection methods for H_2S detection would also help validate the actual blood levels of H_2S. Finally, in contrast to the sulfur ion-selective electrode which detects total sulfur in the blood including its acid labile, bound, or free H_2S forms, the polarographic sensor is sensitive only to freely dissolved H_2S gas. It is possible that a significant amount of H_2S in circulation may not be in a free form as a dissolved gas, offering the rationale for the fact that our blood is not so smelly and the possibility that a polarographic sensor may potentially report a low value. A sensitive "nose" can smell "rotten eggs" in the blood if these eggs are broken, releasing free H_2S gas.

Whereas whether H_2S is a circulating gasotransmitter for both its generation and transportation is still being debated, the paracrine or autocrine effects of H_2S may nevertheless be more critical for regulating the functions of the cells, tissues, and organs where H_2S is produced in the proximity.

Using gas chromatography technique, Furne et al. (194) found very low tissue production of H_2S at nanomolar range in homogenized mouse brain and liver. An interesting comparison for this observation is that Hyspler et al. (260) also used gas chromatography-mass spectrometry (GC-MS) analysis and detected human whole blood H_2S levels at 35–80 μM. Even using a polarographic sensor, others have detected significant tissue production of H_2S from the brain and liver (150).

The detection of the volatile gasotransmitter is already difficult to ascertain and what adds to the challenge is the fact that the safety zone to separate toxicological level and physiological level of H_2S is very narrow. The toxic level of H_2S reported by Warenycia et al. (691) is less than twofold higher than its endogenous level in rat brain tissues. At the time of death of mice exposed to NaHS (60 μg/g), the sulfide concentration in brain, liver, and kidney only elevated from the baseline by 57, 18, and 64%, respectively (407). Comparison between healthy human subjects and age-matched patients with COPD only told a 49.4% increase in serum levels of H_2S with stable COPD (110). This percentage change translates to a H_2S concentration difference of $<$20 μM. This narrowness of the transition zone between physiological/biological and toxicological levels of H_2S can also be found in pharmacological studies where the dose-response relationship of H_2S is relatively steep before a given function change occurred and can quickly cause the opposite effect when H_2S concentration further increased (773). As such, an ideal measurement method for detecting H_2S in mammalians should be sensitive, specific, accurate, noninvasive, on real-time, and require a small quantity of samples.

Many of the current H_2S measurement techniques, such as spectrophotometry, chromatography, and ion-selective electrode, were originally invented to meet the industrial demand for monitoring H_2S pollution in the environment. These techniques are usually invasive and require a bulky quantity of samples. They also do not take account of the conditions for biological studies, such as the existence of H_2S scavenging molecules, interference of hemoglobins or other pigment compounds, redox balance, pH changes, etc.

B. Spectrophotometry Measurement of H_2S

The use of spectrophotometry, also known as the methylene-blue method, to measure trace amounts of H_2S can be traced back to Fischer's study in 1883 for its principle (177) and to the work by Fogo and Popowsky in 1949 for the refining of the technique with the adaption of spectrophotometry (186). This assay is based on the formation of the dye methylene blue when H_2S reacts with ferric chloride ($FeCl_3$) and *N*,*N*-dimethyl-*p*-phenylenediamine (NDPA). Absorbance of the dye in the reaction milieu can be detected by the spectrophotometer. The quantitative relationship (Beer's law) between H_2S concentration and the intensity of the transmitted monochromatic light can then be determined. The minimum detectable concentration of H_2S is determined by the sensitivity of the spectrophotometer to the optical density changes. Photoacoustic spectroscopy of H_2S converted to methylene blue has greater sensitivity than standard spectrophotometric methods. As the acidification is an important component of the methylene blue method, the incorporation of acid-labile sulfide may impact on the interpretation of the actual H_2S level (453).

For animal tissue samples or cells, the methylene blue method has been used often but usually is for detecting the H_2S generation capacity of the samples. In other words, the activity of H_2S-generation enzymes in term of H_2S production rate is assayed, rather than the absolute H_2S concentration. All variations in this application of the methylene blue method are derived from the original 1982 method of Stipanuk and Beck (599). Tissue or cell samples are homogenized and incubated in a reaction mixture. The contents of the mixture are important because including L-cysteine is critical should CSE activity be assayed, but homocysteine should be a component if CBS activity is the goal to examine. This first step is to generate H_2S from samples. Step 2 is to transform H_2S to methylene blue. The generated H_2S at 37°C is trapped with an alkaline zinc acetate solution in an apparatus. Zinc sulfide is formed, precipitated, and subsequently dissolved in a hydrochloric acid solution of *p*-aminodimethylaniline (*N*,*N*-dimethyl-*p*-phenylenediamine). In the presence of ferric chloride, methylene blue is formed. The emitted blue color can be stable for hours and measured at 670 or 650 nm (565, 773). This method can also be adapted to detect sulfate level in water or biological solutions by first reducing sulfate to H_2S with hydriodic and hypophosphorous acids (222).

The application of the methylene blue method to cell-free plasma or other cell-free biological fluids will detect the H_2S already existent, rather than to be generated, since H_2S-generating enzymes are not in the fluid. Therefore, step 1 as described above to maximally activate H_2S-generating enzymes is no longer needed. The fluid sample can be agitated by adding acid to release H_2S into the gas phase, which then interacts with zinc acetate and NPDA to form methylene blue (599). Alternatively, the acid release of H_2S gas and trapping processes are omitted by directly adding NPDA and trichloracetic acid (TCA) to the plasma to directly form methylene blue (206). For H_2S in air samples, the methylene blue method can be modified to use an alkaline solution of cadmium hydroxide to absorb H_2S (27).

C. Nanotube-Based Sensors for H_2S

Electrochemical detection is the most commonly used technology incorporated in compact and portable H_2S gas monitors (624). The principle behind it is the conductivity changes of thin films upon exposure to H_2S gas. Relying on solid state sensors made of semiconducting metal oxides or metals, these portable apparatuses are expensive and suitable for industry utilization. Their drawbacks include high power consumption as found in metal oxide sensors that require high operating temperatures, low sensitivity, short lifetime of often less than 1 yr, and interference by other gases, such as NH_3 and NOx (768).

More popular electrochemical sensors nowadays are based on one-dimensional nanostructures such as bare or functionalized semiconducting single-walled carbon nanotubes (SWNTs) (319, 500), metal oxides, and conducting polymer nanowires (676, 727). Potentially, these sensors may be used to monitor gases with high sensitivity, low sample volume requirement, low power consumption, and low cost (768). A catalytic chemiluminescence sensor made of R-Fe2O3 nanotubes has been developed, which can specifically detect H_2S gas as low as at 10 ppm. The problem with this sensor is that high temperature over 110°C is required for catalytic oxidation of H_2S to occur. It is also not suitable for measuring H_2S in liquid (607). Other sensors based on SnO_2 nanowire (315), In_2O_3 nanowire (356), and ZnO nanowire (673) with increased sensitivity have been reported. The challenges with these one-dimension structures are the difficulties in making the nanostructures and in obtaining large quantities as well as their application under in vivo physiological conditions. CuO-SnO_2 and $ZnSb_2O_6$ have been shown to detect H_2S at concentrations below 1 ppm at 300°C (616).

Using single-wall carbon nanotubes (SWNT) (320) as an H_2S sensor as well as an H_2S carrier has attracted a great attention in recent years. This is because of the adsorption of H_2S by activated carbon and the realization of the structural advantages of the carbon nanotubes, which are the

uniform pore size distribution, high surface area, and excellent electronic properties. High surface area will result in an increased amount of irreversibly adsorbed H_2S. The activated carbon facilitates H_2S reaction with oxygen at low temperatures, leading to the production of sulfur and water (312). SWNT-based H_2S biosensor will also potentially reduce the sample volume to nanoscale.

The initial attempt of using multi-wall carbon nanotubes to measure H_2S in solution was made by Wu et al. (722). After carbon nanotubes are immersed in a H_2S solution, on the contact interface between carbon nanotubes and H_2S solution formed is a thin water film. Oxygen molecule is also dissolved in the film and adsorbed by the carbon nanotubes. Carbon nanotubes also absorb H_2S by the van der Waals force. The interaction of H_2S (hydrosulfide ions and protons) and oxygen on the nanotubes forms hydroxyl ions and sulfur. The protons neutralize the hydroxyl ions and produce water. But the spectra of fluorescence of sulfur on carbon nanotubes can be assayed with either a Raman or a confocal laser scanning microscope (720). It was found that fluorescence intensity was increased, closely correlated with the increased concentrations of H_2S in the solution. In this preliminary study, 10 μM H_2S in water was successfully measured (722).

To take one step further toward the biological application of the carbon nanotube-based H_2S biosensor, Wu et al. (721) applied this carbon nanotube fluorescence technique to measure H_2S level in serum and reported that the binding of H_2S to nanotubes was not affected by the presence of proteins in rat serum. After removing endogenous H_2S in the serum with hemoglobin, exogenous H_2S added to the serum was successfully detected with a linear relationship between H_2S concentrations (20, 50, and 100 μM) and fluorescence intensities. The mechanism for using carbon nanotubes to detect H_2S, even in the presence of proteins, is believed to be due to a continuous serum albumin film formed on the surface of carbon nanotubes. Other proteins or large molecules cannot pass the albumin film, but H_2S can easily move and pass through this film to the surface of carbon nanotubes.

What Wu and co-workers (720–722) did is the combination of carbon nanotube adsorption with the fluorescence emission detection, a chemical approach. A different strategy by detecting the conductance change of carbon nanotubes after binding with H_2S was taken, an electrical approach (768). The principle for this strategy is to conduct site-specific electrodeposition of gold nanoparticles on SWNT networks. The adsorption of H_2S molecules at different concentrations onto the gold nanoparticle surface can change the carbon nanotube conductivity to different degrees. The researcher reported superior sensitivity of these nanostructures toward H_2S at room temperature with a detection limit of 3 ppb. The application of these nanostructures for detecting H_2S in liquid preparation and biological samples has not been reported.

D. Sulfur Ion-Specific Electrodes

Sulfur ion-specific electrodes have been frequently used in detecting H_2S level in blood and cell culture media. The method is easy to operate, and the initial setup is of low cost. Typically, the ion-specific electrode has a linear response range of between 0.1 M and 10 μM and a detection limit on the order of 1–10 μM. The observed detection limit is often affected by the presence of other interfering ions or impurities. With a modified sulfide-specific electrode, Searcy and Peterson (549) reported measurement of very low free sulfide concentration (0.5 μM). This measurement was done with continuous injection of Na_2S solution into the sample chamber to maintain a constant concentration. Its application to biological fluids close to physiological conditions is not clear.

Sulfur ion-specific electrodes are sensitive only to S^{2-}, and as such, free H_2S needs to be fully dissociated. This can be achieved under a strong alkali conditions and with a complete lack of oxidation (205). For both blood (whole blood, serum, or plasma) and cell culture media, this alkali and antioxidant condition might cause protein desulfuration, and the electrodes may detect S^{2-} dissociated from H_2S and released from proteins. Furthermore, using the electrodes still requires bulky samples and is an off-line measurement.

E. Polarographic H_2S Sensor

A novel polarographic H_2S sensor (PHSS) was developed in 2005 as a voltammetry, containing anode, cathode, and electrolyte protected from solution constituents by an H_2S-permeable polymer membrane (150). The application of PHSS has been reported at cellular, tissue, and organ levels with the claimed high sensitivity at the nanomolar range and rapid response time to H_2S. Real-time measurement of the levels of H_2S and O_2 in respirometry and vessel tension experiments with PHSS has been achieved (313). Most of PHSS have the dimensions similar to that of the polarographic oxygen sensor. Recent advance sees the availability of the miniature PHSS for real-time measurement of H_2S production in biological samples. It was reported that the miniature PHSS detected H_2S production by brain supernatants at $\sim$10.6 pmol·s^{-1}·mg protein^{-1} (246), which is significantly higher than that in vascular tissues (0.5–1.1 pmol·s^{-1}·mg protein^{-1}) (245, 773). Just like the real-time polarographic sensors for other gas molecules (O_2, NO, or CO), however, to have consistent and reliable reading of H_2S level with commercially available PHSS is more often than not a daunting challenge and a frustrating experience.

F. Chromatography Analysis of H_2S

Chromatography includes gas chromatography, liquid chromatography, ion-exchange chromatography, affinity chromatography, and their variations such as HPLC (high-performance liquid chromatography or high-pressure liquid chromatography). The readers are referred to a thorough review by Ubuka (650), which detailed the application of chromatograph technology in H_2S detection. In short, liquid chromatographic determination of sulfide with or without derivatization and ion chromatography of sulfide have been conducted. HPLC analyses of sulfide after conversion to methylene blue, to thionine, or to the monobromobimane derivative or after labeling with *o*-phthalaldehyde (OPA) have been reported. Gas chromatography has also been employed to analyze sulfur compounds in air, aqueous, and biological samples (650). For example, the measurement of H_2S in air by ion chromatography has the working range of 20–500 μM for a 20-liter air sample (96). Gas chromotography-mass spectrometry has been used to detect H_2S in animal tissues based on the amount of trapped S^{2-} after acidification of H_2S (194, 260).

Reverse-phase (RP)-HPLC for the determination of H_2S-derived methylene blue was used in measuring the sulfide content in brain, liver, and kidey from sulfide-treated mice. After exposure of mice to 60 μg/g Na_2S, tissue contents of H_2S were all significantly increased (407). Shen et al. (556) reported in 2011 a novel and sensitive method to detect physiological levels of free H_2S in cell lysates, tissue homogenates, and body fluids. This method is built on the rapid reaction of monobromobimane with H_2S under basic conditions at room temperature to produce sulfide-dibimane (SDB). SDB is stable, a feature to favor over the unstable H_2S for biological assays. SDB is also more hydrophobic than most physiological thiols. RP-HPLC can separate SDB with a gradient elution and then analyze it by fluorescent detection. The sensitivity of this SDB-based RP-HPLC analysis reaches the H_2S level as low as 5 nM, which is in sharp contrast to the methylene blue-based spectrophotometry method which has a low limit of 2 μM (556). When the SDB-based method was applied to wild-type mice, heterozygous CSE knockout (CSE HT) mice, and homozygous CSE KO mice, clear differentiation in plasma level of H_2S was achieved. CSE HT mice have lower plasma level of H_2S than that of wild-type mice, but higher level than that of CSE KO mice (556).

Sensitive and selective detection of H_2S has been one of the hot spots as well as one of the bottlenecks in H_2S study. New methodologies are being continuously devised and reported and the existing methods improved and adapted to new applications. The quick oxidation and scavenging of H_2S in biological samples are the biggest challenges for accurate and rapid measurement of H_2S levels. At this moment, the spectrophotometry-based method is still of the choice to determine tissue or cell production of H_2S, whereas sulfur ion-specific electrodes and polarographic H_2S sensors hold potential for real-time measurement of H_2S net levels in blood or other body fluids. For analyzing H_2S in air samples, such as exhaled air from lungs, chromatography analysis of H_2S would be more suitable. Furthermore, the fluorescence-based quantitative or semi-quantitative methods would be useful for detecting H_2S production in specific cellular organelles.

XIV. CONCLUSIONS AND PERSPECTIVES

The exploration of the physiological importance of H_2S over the last decade or so has led to many unequivocal conclusions and significant advances on H_2S biology and medicine. We know by now, without any doubt, that most types of cells in our body, if not all, possess H_2S-generating systems, from different types of enzymes to their substrates. Cellular H_2S production and metabolism are tightly and precisely regulated depending on cell types and metabolic particulars. Endogenously produced H_2S exerts profound impacts on physiological functions at cellular, tissue, system, and whole body levels. These acts of H_2S are mediated by different molecular targets, such as different ion channels and signaling proteins. In addition to its direct interaction with thiol-containing proteins, H_2S also affects other signaling processes. Redox balance is one example of these processes. Alterations of H_2S metabolism lead to an array of pathological disturbances in the form of hypertension, atherosclerosis, heart failure, diabetes, cirrhosis, inflammation, sepsis, neurodegenerative disease, erectile dysfunction, and asthma, to name a few. By directing endogenous H_2S metabolism or applying exogenous H_2S, we may find novel solutions for preventing, interfering, and treating a wide spectrum of diseases.

The conclusions do not rebuff controversies. The advances do not mitigate challenges. A long journey of explorations does not signal the end of the trudge. In this context, the 10 most controversial and challenging issues in H_2S research are presented and the 10 most promising future directions are suggested.

A. Controversial and Challenging Issues in H_2S Research

1) In many cases H_2S acts as a double-edged sword, offering opposite impacts on the given biological processes. The examples for these biological processes include inflammation, diabetes, contractile responses, and cancer development, etc. Buga et al. (78) showed that two-day poststroke exposure of aged rats to H_2S caused deep hypothermia and a 50% reduction in infarct size without obvious neurological deficits or physiological side effects. But administration of NaHS by Qu et al. (502) significantly increased the infarct volume in a rat stroke model created by permanent occlusion of the middle cerebral artery. Administration of H_2S-generating enzyme inhibitors reduced infarct volume. NaHS decreased the viability of colon cancer cells (WiDr cells) (93), but in another study NaHS induced proliferation of HCT

116 cells and SW480 cells (87). As these studies used immortal cell lines, the role of H_2S in cancer development in vivo is uncertain. Another example for the opposite effects of H_2S is inflammation. Both pro- and anti-inflammatory roles of H_2S have been reported. H_2S activates NF-κB in pancreatic acinar cells to promote inflammation (617, 618, 776). H_2S inhibits NF-κB in HUVECs to suppress inflammation (690). Cell type difference aside, the admission routes and H_2S releasing speed for the H_2S donors may also be accountable for the controversial reports.

2) Whether endogenous H_2S is important in specific types of cells under physiological conditions is not clear. In the heart, mRNA of CSE is detected under physiological conditions (160, 206, 738, 778), and the application of CSE inhibitor PPG significantly decreased H_2S production from the heart (251). Under ischemic conditions (778) or after overexpression of CSE in the heart (160), CSE protein was clearly detected with Western blot analysis. However, no CSE protein has been unequivocally detected in the heart under physiological conditions (270). This may result from the limited availability of species- and tissue-specific antibodies against the given H_2S-generating enzyme. It may also argue that endogenous H_2S is not needed in the heart under physiological conditions so that CSE protein serves on an on-call basis, translated from mRNA at meaningful amount under emergency situation.

3) The enzymatic basis for H_2S production in different types of cells is not always clear and consistent in the literature. A case in point is the pancreas. Both CBS and CSE have been detected in pancreas, but association of different cell types in the pancreas with CBS and CSE becomes puzzling. Kaneko et al. (295) reported that "CBS was ubiquitously distributed in the mouse pancreas, but CSE was found only in the exocrine. Freshly isolated islets expressed CBS, while CSE was faint." Wu et al. (717) reported high expression level of CSE in rat pancreatic islets, but CBS mRNA expression level was extremely low.

4) Functional changes associated with the altered expression level of H_2S-generating enzymes had been directly related to the role of endogenous H_2S in some studies. Unfortunately, these studies had not directly monitored the changes of H_2S level and disregarded potential contributions from other molecules. As H_2S is only one of many molecules involved in trans-sulfuration and reverse trans-sulfuration pathways, changes in H_2S-generating enzymes may affect many other molecules, not only the products of these enzymes but also their substrates. Among these products and substrates, in addition to H_2S, are pyruvate, ammonium, thiosulfate, cysteine, homocysteine, cystathionine, α-ketobutyrate, and α-ketoglutarate, etc.

5) The use of knockout mice generates different phenotypes. This emphasizes the importance of genetic background for interpreting the biological role of a given enzyme. Yang et al. (738) showed that CSE deficiency in knockout mice led to a hypertension phenotype. However, Ishii et al. (269) reported that their CSE-KO mice displayed a phenotype of paralysis of the upper extremities and skeletal muscle atrophy after feeding with a low-cysteine diet. On the other hand, these mice were not hypertensive. This discrepancy may be related to the difference in genetic background of the mice used in these two studies (mixed C57BL/6J $\times$ 129SvEv versus C57BL/6J).

6) Manipulating the expression or activity of the H_2S-generating enzymes can be misinterpreted if not cautious enough. Using pharmacological tools to inhibit enzyme activity (e.g., PPG for CSE or AOA for CBS) may nonspecifically affect other proteins (especially at high concentrations) or do not actually inhibit the enzymes (especially at low concentrations). Gene expression manipulation would be an alternative approach to circumvent the requirement for pharmacological inhibitors. The expression of H_2S-generating enzymes can be downregualted or upregulated at the level of transcription and translation. Certainly, the availability of CSE- or CBS-gene knockout animals invites many applications for examining the impact of the targeted genes after their gene transcription is completely disrupted (738). Beyond this gene manipulation at the whole animal level, there are other approaches at the cellular or tissue levels.

Gene-specific short interfering RNA (siRNA) is one technique often used to silence gene expression at the tissue or cell levels (76, 739). This technique introduces synthesized double-stranded RNAs into a diverse range of cells to degrade the target mRNA. It minimizes off-target and nonspecific effects, providing a short-term gene silencing. The efficiency of transfection and the completeness of silencing the target gene, relating to the utilization of different transfection agents and different types, are two major challenges for siRNA technique. CSE-specific siRNAs, for example, cannot completely knockdown CSE expression, and certain side effects of the transfection agents would also need attention (76, 739). It is perceivable to circumvent these problems by employing short hairpin RNA (shRNA) or antisense oligonucleotide techniques. As a different RNA interference means, shRNA transfection aids the integration of the shRNA cassette into the genome and generates stable knockdown, compared with the transient knockdown by siRNA technique, of the target mRNA (60). Synthetic antisense oligonucleotides are small single-stranded DNA or RNA fragments recognized by Watson-Crick base pairing. The antisense RNA can prevent the protein translation, whereas antisense DNA can form specific moieties with the complementary RNA. Such moieties will be then irreversibly degraded by RNase H. However, the control of transfection efficiency of antisense oligonucleotides is also a tedious task and the antisense oligonucleotides can also be

degraded by endonucleases (156). Neither antisense oligonecleotides nor shRNA have been used to knockdown the expression of H_2S-generating enzymes to date.

Adenovirus vectors and cDNA plasmids have been used to upregulate CSE gene expression (553, 608, 732). The noticeable advantages of adenovirus-mediated gene transfer include its safety and high efficiency for transfecting both replicating and differentiated cells (208). One of the shortfalls of this approach is the low transfection efficiency at the tissue or whole animal levels. Since adenovirus only remains as an episome in the nucleus and would not integrate into the cell chromosome, the transgene expression is transient and can only last several days to weeks (208). Another major concern on adenovirus transfection is that the viral proteins can be expressed in the host and cause an immune response, which can hamper the transduction efficiency of adenovirus. Plasmid DNA-mediated CSE gene transfer other than immunogenic viral vectors is often used for transgene expression (553, 732), but plagued by high variability and low efficiency. Its advantage, however, is manifested as being relatively nonimmunogenic.

At the whole animal level, CSE transgenic mice have been produced in which CSE gene was conditionally overexpressed in cardiomyocytes by using a construct consisting of the α-MHC promoter (160). Heart-specific CSE transgenic mice allow the researchers to observe experimentally what happens to an entire organism during the progression of heart disease.

7) The actual concentration of H_2S in blood has been unclear. As the micromolar level of H_2S has been detected in many reported studies; much lower H_2S concentrations at nanomolar range or below the detection threshold have been reported in other studies. As discussed in section XIII, this discrepancy can be attributed to the sensitivity and reliability of different measurement methods, but could also reflect the existence of different forms of H_2S in circulation, free versus bound, as well as the kinetics of H_2S metabolism.

8) H_2S poisons mitochondrial respiratory chain by binding to the iron of cytochrome *c* oxygenase (439), but it also helps reduce mitochondrial damage and offers cytoprotection. Not only the concentration of H_2S directs different mitochondrial outcomes, where and how H_2S is produced inside the cell may also be accountable. It may also be asked whether H_2S is needed for normal mitochondrial function or whether it is only needed when oxygen supply is insufficient.

9) Identification of the molecular targets of H_2S has been a challenge. K_{ATP} channels in many cells are responsible for the effects of H_2S (773), but in other cells, H_2S seems not to act on the same channels. Increased, decreased, or no change in the activities of calcium channels in the presence of H_2S have all been reported (622). Moreover, the mediating role of K_{ATP} channels on H_2S effects has been studied in many cases based on the application of exogenous H_2S donors at relatively high concentrations or the interaction of these donors with K_{ATP} channel blockers. As such, the physiological or pathophysiological relevance of these studies needs to be carefully evaluated. Direct electrophysiological recording of K_{ATP} channel currents in the absence and presence of endogenous H_2S should be more meticulously conducted, which can never be replaced or extrapolated by the observations of cellular functional changes (such as cell proliferation or tissue contraction) after the application of K_{ATP} channel blockers. Whether H_2S affects cGMP/cAMP is also perplexing. Cai et al. (88) reported that cAMP or cGMP levels were not affected by NaHS, but Bucci et al. (76) showed that H_2S increased cAMP and cGMP levels by nonselectively inhibiting phosphodiesterases (76).

10) Is H_2S an antioxidant or pro-oxidant factor? H_2S inhibits oxidative stress by increasing antioxidative GSH content (340, 341) or by reducing the production of H_2O_2, $ONOO^-$, and O_2^- in the presence of homocysteine (730). On the other hand, H_2S may transfer electrons to other molecules in the cells. The formation of sulfhydryl radicals (HS^*/S^{*-}) from NaHS has been demonstrated in vitro in protolytic and aprotic solvents (592) or in the presence of peroxidase and H_2O_2 (438). Moreover, one of the consequences of protein *S*-sulfhydration induced by H_2S is the production of persulfides. Persulfides themselves can mediate the conversation of molecule oxygen to reactive oxygen species under physiologically relevant conditions (103).

B. Promising Future Directions in H_2S Research

1) Enlarging the search scope for the physiological importance of H_2S to include many unexplored organs and systems as well as diseases. For example, H_2S may play an important role in the regulation of immune functions, which encompasses many infectious diseases. Acquired immunodeficiency syndrome (AIDS) patients have altered sulfur metabolism with significantly decreased cysteine and glutathione content in liver (645). Elucidation of the changes in the expression and activity of H_2S-generating enzymes in this disease and the effect of H_2S on human immunodeficiency virus (HIV) replication may shed light on the novel H_2S-based therapeutic to this virus infection and others. Let's take metabolic syndrome as another example (99). Sulfide poisoning has been suggested to induce metabolic syndrome. H_2S participates in the pathogenesis of hypertension and diabetes, two abnormalities of metabolic syndrome. Obesity is also part of the metabolic syndrome, and its development and prognosis may also be linked to H_2S metabolism. In fact, it has been shown that obese patients have lower plasma H_2S levels (706). Whether the altered H_2S metabolism is the cause or the consequence of obesity is not yet clear.

2) Interaction of H_2S with other gasotransmitters. Both H_2S and NO modify sulfhydryl groups but with different mechanisms. Once a sulfhydryl group is *S*-sulfhydrated by H_2S, its reaction to NO through *S*-nitrosylation may no longer be the same, and vice versa. This cross-interaction would determine the net structural and functional changes of the involved protein. For a protein with multiple sulfhydryl groups, the situation becomes even more intriguing as to which specific sulfhydryl group and how many groups would be the target of H_2S or NO or both. The kinetics and dissociation efficiency of *S*-sulfhydration and *S*-nitrosylation in the same reaction system are also important factors for directing the interaction between H_2S and NO. Beyond the interaction on the common target, how the production and metabolism of H_2S are affected by NO and CO, and vice versa, should be carefully dissected out. Furthermore, both NO and CO interact with heme, and CBS is a heme-protein. As such, these three gasotransmitters may all be involved in heme-related biological processes. More details of these interactions are discussed in section VII.

3) Ways to selectively alter H_2S level in different tissues. Because H_2S effects are highly tissue-type specific, increasing H_2S level may be beneficial to one tissue but detrimental to the others. It would be preferable to devise tissue-type or cell-type targeted strategies and approaches to alter local H_2S level.

4) Pharmacological advances in developing H_2S donors and their clinical applications. The releasing speed and amount of H_2S from different donors should be controllable. Scavengers of H_2S and inhibitors of H_2S-generating enzymes with improved specificity and potency will also find their clinical applications. The administration routes of these compounds should be diversified to include inhalation, injection, skin patch, or oral uptake, to maximize their potency and safety and to decrease side effects.

5) Role of H_2S as an oxygen sensor and the underlying mechanisms. This research would affect multiple organs and systems in our body with complex mechanisms. With different oxygen levels, *a*) the transition of H_2S from its bound form to free form may be changed; *b*) the oxidation of H_2S may alter the nature of the sulfur signal and as such sulfane sulfur may be involved; *c*) H_2S may differentially modulate ATP production rate in mitochondrial; *d*) H_2S may alter ATP utilization in the cytosol; and *e*) H_2S may either amplify or counteract a given functional event, such as turning a vasoconstriction into a vasodilation.

6) H_2S and population study. Not only genetic makeup of different populations may be different, but epigenetic changes may also become population traits. Genetic deficiency of H_2S-generating enzymes in different human populations should be mapped out. Altered phenotype or gene expression profile related to abnormal H_2S metabolism may also be associated with some populations.

7) H_2S and nutrition, diet, exercise, and gender difference. Healthy diet and nutrition provides our body with the needed H_2S in the vehicles of garlic, eggs, broccoli, or fermented fish, etc. The effects of exercise on endogenous H_2S levels or the effect of H_2S on body fitness are both intriguing. Deficiency of CSE in mice leads to hyperhomocysteinemia much more in females than in males (738). Further studies devoted to H_2S-related gender difference and the interaction of H_2S with estrogen are warranted.

8) Evolutionary significance and impacts of H_2S metabolism. Because the metabolism of H_2S may be universal for all phyla or phylum of animals, plants, fungus and bacteria, studies on the physiological importance of H_2S in vertebrate and invertebrate would be aspiratory and insightful for identifying the biological importance of this gasotransmitter in other life forms. Thus studies on the role of H_2S in plant growth, seeding, flowering, and responses to environmental and seasonal changes are due. Another example is the relationship between evolutionary change of H_2S metabolism and environmental stress. A recent study shows that the neotropical fish *Poecilia mexicana* exhibits strong site-specific life-history divergence as an adaptation strategy to respond to toxic H_2S level exposure (516).

9) Integration of the studies on H_2S physiology and H_2S toxicology. Occupational and environmental health concerns of H_2S, i.e., H_2S toxicology, were examined far before the study on H_2S physiology. However, these two schools appear not to dance together as they should. Sharing the expertise base and data bank will benefit both schools from enlarging research capacity, developing more sensitive detection devises, determining the safety threshold of H_2S to inventing therapeutic scavengers of H_2S, etc.

10) Development and improvement H_2S detection technology and methodology. This task is not really easy due to the gas nature of H_2S, and the same challenge also applies to other gasotransmitters. However, the completion of this task will be greatly rewarded with establishment of the precise roles of H_2S in different systems and under different conditions.

The search is ongoing. To be continued . . .

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